410 Rec'd PCT/PTO 14 JUL 2000

FORM PTO-1390 (Modified) (REV 11-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **6137.P US**

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR: 09/600392

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US99/00371	**12 January 1999 (12.01.99)**	**16 January 1998 (16.01.98)**

TITLE OF INVENTION

AN AUTOREGULATORY SYSTEM FOR VALIDATING MICROBIAL GENES AS POSSIBLE ANTIMICROBIAL TARGETS USING A TETRACYCLINE-CONTROLLABLE ELEMENT

APPLICANT(S) FOR DO/EO/US

Cheryl L. Quinn et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☐ has been transmitted by the International Bureau.
   - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☐ have been transmitted by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

Communication Regarding Sequence Listing (1 pg.); Paper copy of Sequence Listing (15 pgs.); Sequence Listing in computer readable form; transmittal document (in triplicate); and itemized return receipt postcard.

"EXPRESS MAIL" MAILING LABEL NUMBER: EL518337622US

DATE OF DEPOSIT: 14 July 2000

I HEREBY CERTIFY THAT THIS PAPER OR FEE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE "EXPRESS MAIL POST OFFICE TO ADDRESSEE" SERVICE UNDER 37 CFR 1.10 ON THE DATE INDICATED ABOVE AND IS ADDRESSED TO THE ASSISTANT COMMISSIONER FOR PATENTS. WASHINGTON, D.C. 20231

PRINTED NAME: Anne M. Richards

SIGNATURE: Anne M. Richards

PCTUS1/REV03

534 Rec'd PCT/PTO 14 JUL 2000

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 09/600392	INTERNATIONAL APPLICATION NO. PCT/US99/00371	ATTORNEY'S DOCKET NUMBER 6137.P US

21. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS	PTO USE ONLY
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$970.00**		
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but Internation Search Report prepared by the EPO or JPO	**$840.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	**$690.00**		
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$670.00**		
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)	**$96.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =		**$840.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	77 - 20 =	57	x $18.00	**$1,026.00**	
Independent claims	6 - 3 =	3	x $78.00	**$234.00**	
Multiple Dependent Claims **(check if applicable).**			☐	**$0.00**	
	TOTAL OF ABOVE CALCULATIONS =			**$2,100.00**	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable).**			☐	**$0.00**	
SUBTOTAL =				**$2,100.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$2,100.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).**			☐	**$0.00**	
TOTAL FEES ENCLOSED =				**$2,100.00**	
				Amount to be: refunded	$
				charged	$

☒ A check in the amount of **$2,100.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. in the amount of to cover the above fees. A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **13-4895** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Mueting, Raasch &Gebhardt, P.A.
P.O. Box 581415
Minneapolis, Minnesota 55458-1415
United States of America
ATTN: Ann M. Mueting

Ann M Mueting

SIGNATURE

Ann M. Mueting

NAME

33,977

REGISTRATION NUMBER

July 14, 2000

DATE

AN AUTOREGULATORY SYSTEM FOR VALIDATING MICROBIAL GENES AS POSSIBLE ANTIMICROBIAL TRAGETS USING A TETRACYCLINE-CONTROLLABLE ELEMENT

Field of Invention

Methods for identifying which microbial genes are targets for inhibition by antibiotics. Specifically a tetracycline-regulated system which provides autoregulatory, inducible gene expression in recombinant microbes, such as bacteria, and in animals infected with the microbes, such as bacteria, is described.

Background of the Invention

The development of widespread antibiotic resistance in microbial pathogens has created an urgent medical need for new antimicrobial agents. Instead of relying on derivatives of existing antimicrobial agents, the pharmaceutical industry is looking for novel microbial processes to target in an attempt to create new classes of compounds (Knowles, D. J. C., *Trends in Microbiol.*, 1997,5:379-383).

Genes essential for maintaining an infection in an animal or essential for growth of the pathogen *in vitro* are good targets for antibiotic development. Traditionally, "essential genes" have been prioritized as good antimicrobial targets. Essential genes are those required for microbial cell growth *in vitro* and include such genes as those encoding DNA gyrase, ribosomal subunits, and cell wall biosynthetic enzymes. Many of these proteins and cell components have been identified as being encoded by essential genes because there are classic antimicrobial agents shown to inhibit the products of these genes (quinolones, tetracyclines, and "beta"-lactams respectively). Other essential genes have been identified from the characterization of conditional lethal mutants.

With the availability of whole microbial genome sequences, there are now many previously unknown and uncharacterized genes available which may turn out to be essential. The conventional approach for testing if a gene is essential is to attempt making a construct of that organism where the test gene is deleted or inactivated. If the organism can survive with the gene deleted or inactivated, the gene is not considered essential. For example, see Stranden, A. M., Ehlert, K., Labischinski, H., and Berger-Bachi, B., 1997, *J. Bacteriol.* 179:9-16). However, failure to create a mutant organism with an inactivated or deleted gene does not always mean that the gene is essential. For example see, Okada, K., Minehira, M.,

Zhu, X., Suzaki, K, Nakagawa, T., Matsuda, H. and Kawamukai, M., 1997, *J. Bacteriol.* 179:3058-3060. This negative proof for a conclusion may not always be valid. There may be other reasons why the gene deletion or inactivation could not be made.

Recently, virulence factors and genes required for pathogenesis have been suggested as novel targets for antimicrobial agents. Two widely read and referenced techniques, signature tagged mutagenesis (STM; Hensel, M., Shea, J. E., Gleeson, C., Jones, M.D., Dalton, E. and Holden, D., 1995, *Science* 269:400-403) and *in vivo* expression technology (IVET; Mahan, M. J., Tobias, J. W., Slauch, J. M., Hanna, P. C., Collier, R. J., and Mekalanos, J. J., 1995, *PNAS* 92:669-673) allow scientists to quickly identify a number of bacterial genes required for pathogenesis or that are induced during host infection. While these genes represent good targets for developing attenuated strains for vaccines, it is not clear if they represent valid targets for inhibition by antimicrobial agents. The critical distinction in this evaluation of potential gene targets is that antimicrobial agents are used to inhibit microbial pathogens after infections are established. If virulence factors or pathogenicity genes are only required to establish the infection, inhibition of these in an established infection would not clear the infection. If, after stopping the synthesis of specific genes, an established infection is cleared, those specific genes are essential for maintaining the infection. Therefore, it would be advantageous to develop a method for turning off an endogenous gene to test if it is essential for growth. Such a method would facilitate the identification of antimicrobial targets which should speed the development of new classes of antimicrobial compounds.

Many of the ideas concerning such systems have been disclosed, see the definitions, theories and descriptions of PCT application PCT/US96/07937, International Publication Number WO 96/40979, published 19 December 1996 (19.12.96). PCT/US96/07937 is hereby incorporated by reference into this document; however, recombinant sequences and the examples disclosed in PCT/US96/07937 are NOT incorporated here.

Also US 5,464,758 disclose many of the mechanisms of the tetracycline-Responsive Promoters. US 5,464,758, published 7 Nov. 1995 is incorporated in part here, the general definitions, theories, principles, concepts, general information about

the tetracycline operator (*tetO*) sequences is incorporated into this document by reference but the sequences disclosed in US 5,464,758 are NOT incorporated into this document. Here Applicant describes a system that works.

Summary of the Invention

This invention provides for a process that allows the characterization of a microbial gene or genes, where the gene encodes a gene product; where the gene product is a gene target; where the gene target is important to a microbe's ability to infect or sustain an infection in a mammal, where the microbe is: genetically altered to become a genetically altered microbe, such that the amount of the gene product produced by the genetically altered microbe is regulated and controlled by a Tetracycline-Controllable Element or TCE; where the TCE is a gene regulatory system that controls the expression of the target gene or gene product, through its ability to modulate the function of the gene in response to the microbe's exposure to tetracycline, and where the TCE is comprised of a tetracycline-controllable transcription promoter polynucleotide sequence; where the gene, which may be any gene which encodes a microbial protein, or more generally a microbial gene product, is regulated by the TCE such that the gene produces either greater or lesser amounts of gene product, depending upon whether or not the genetically altered microbe is exposed to tetracycline; where the mammal is a plurality of at least two or more mammals, where the mammals are initially exposed to tetracycline and infected with the genetically altered microbe; followed by: the removal of the tetracycline exposed to a portion of the mammals, such that at least one or some mammals **of** one group of the mammals are exposed to tetracycline and the other one or group of mammals are not exposed to tetracycline; followed by: a comparison of the degree of infection, microbe levels, or physiological condition of the mammals exposed to tetracycline, compared to the degree of infection, microbe levels, or physiological condition of mammals not exposed to tetracycline; followed by: the identification of the genes, important to a microbe's ability to infect or sustain an infection in a mammal, where the comparison of the mammals exposed to tetracycline compared to the mammals not exposed to tetracycline shows a meaningful difference between the two groups of animals, or the infection levels of those animals.

In related aspects of the invention the TCE is a gene regulatory system that controls the expression of the target gene or gene product, through its ability to modulate the function of the gene in response to the microbe's exposure to tetracycline, and where the TCE is comprised of a tetracycline-controllable transcription promoter polynucleotide sequence, operably linked to a polynucleotide sequence encoding a reporter gene, the tetracycline-controllable transcription promoter polynucleotide sequence, is a prokaryotic transcription promoter, that may be operably linked to a polynucleotide sequence encoding a reporter gene (RG) and a target gene (TG). The reporter gene can be β-lactamase. The microbe can have additional genetic alterations comprising a tetracycline resistance (or protection) and repressor DNA cassette (TRRDC). The TCE, the TRRDC, the RG, and the TG can all be on the same DNA cassette, which may be referred to as a Regulatory DNA Cassette or RDC, but the other components beyond the TCE are not required to be on the RDC. The TRRDC can comprise the structural gene *tetM*, a tetracycline resistance gene, the structural gene *tetR* , a tetracycline repressor gene and it can have a promoter operably linked to the TCE.

A meaningful difference between the two groups of animals being tested is a mathematically significant difference in the survival rates or the levels of microbes, or levels of infection present in the mammals. The meaningful difference between the two groups of animals is a mathematically significant difference in the survival rates of the groups of animals. The the significant difference in the survival rates of the groups of animals shows that animals exposed to tetracycline have poorer health, higher rates of infection, lower survival or higher levels of microbes than animals not exposed to tetracycline. The animals can be mammals, preferably mice or other rodents.

The tetracycline resistent gene of the TRRDC can be comprised of sequences from the *Staphylococcus aureus tetM* gene. The tetracycline repressor gene of the TRRDC can be derived from the Tn10 transposon.

The microbe can be a recombinant bacterium. It can be a Staphylococcus species, such as *Staphylococcus aureus*, or a virus, a lower eukaryote, or even a yeast.

The invention further comprises an isolated DNA molecule for integrating a heterologous polynucleotide sequence at a pre-determined location in a prokaryotic

chromosome to operably control an endogenous prokaryotic gene, the DNA molecule comprising recombining element (RE) and a tetracycline controllable element (TCE), the TCE comprising a tetracycline-controllable prokaryotic transcription promoter polynucleotide sequence flanked at its 5' end by the RE, the RE comprising additional polynucleotide sequences of sufficient length for homologous recombination between the isolated DNA molecule and the prokaryotic chromosome.

This isolated DNA molecule can have a polynucleotide sequence encoding a reporter gene operably linked to the TCE. The reporter gene can be beta-lactamase. In some cases at least one prokaryotic transcription terminator polynucleotide sequence positioned between the RE and the TCE. The DNA can also have a polynucleotide sequence encoding a prokaryotic tetracycline resistance protein operably linked to a prokaryotic transcription promoter polynucleotide sequence positioned between the RE and the TCE. The tetracycline resistance protein can be derived from the *Staphylococcus aureus tetM* gene. The DNA can have a polynucleotide sequence encoding a prokaryotic tetracycline repressor protein operably linked to a tetracycline-controllable prokaryotic transcription promoter polynucleotide sequence positioned between the RE and the TCE. The tetracycline repressor may be a Tn10 transposon, derived from a Tet repressor. Sequences of Tn10 transposons are disclosed herein. Associated vectors and cells, especially prokaryotic host cells are described. The DNA has various recombining elements and tetracycline-controllable elements, reporter genes like beta-lactamase whose sequences that may be selected from the sequence listing.

The DNA molecules herein can be operably inked to a reporter gene, such as beta-lactamase (β-lactamase), especially a beta-lactamase from the included sequence listing, and the reporter gene can be operably linked to the tetracycline-controllable element.

The tetracycline resistance protein can be derived from the *Staphylococcus aureus tetM* gene or from various sequences provided. The tetracycline repressor may be a *tetR* gene derived from the Tn10 transposon, and several sequences are provided. At least one prokaryotic transcription terminator seqeuence can be positioned between the tetracycline-controllable element and one or more recombining elements. A prokaryotic tetracycline resistance protein can be operably linked to a

transcription promoter polynucleotide sequence. A polynucleotide sequence encoding a tetracycline repressor protein can be operably linked to a transcription promoter polynucleotide sequence. The DNA desribed here can be made into a form suitable for transformation of a host cell.

The invention further comprises another different type of isolated DNA molecule for integrating a heterologous polynucleotide sequence at a pre-determined location in a prokaryotic. This other type of DNA can be described as: an isolated DNA molecule for integrating a polynucleotide sequence including tetracycline-controllable elements (TCE) at a pre-determined location in a target DNA molecule, the isolated DNA molecule comprising the following DNA elements fused in sequence: a) a first prokaryotic transcription terminator polynucleotide sequence; b) a second prokaryotic transcription terminator polynucleotide sequence; c) a polynucleotide sequence encoding a prokaryotic tetracycline resistance protein; d) a polynucleotide sequence encoding a prokaryotic repressor protein; e) a first tetracycline-controllable prokaryotic transcription promoter polynucleotide sequence; f) a second tetracycline-controllable prokaryotic transcription promoter polynucleotide sequence; and g) a polynucleotide sequence encoding a reporter protein; the isolated DNA molecule comprising a polynucleotide sequence including the TCE flanked at the end opposite the polynucleotide sequence encoding the reporter protein by additional polynucleotide sequences of sufficient length for homologous recombination between the isolated DNA molecule and the target DNA molecule at a pre-determined location. All of the modifications described above can be applied to the DNA molecule described in this paragraph. This DNA molecule may also be described as a DNA cassette, it may also be called an RDC. Note an RDC does not have to be on a single cassette, the elements of an RDC can be fashioned in many different ways. Elements of the RDC can even be taken from the microbe itself.

Finally, this system is describe in detail with bacterial it can also be adapted to other type of organisms. When the system is used with a virus, eukaroyte or yeast the transcription promoters and structural genes should be modified in a manner apparent to one skilled in the art that would make the promoters and genes active in that organism.

Brief Description of the Figures

Figure 1 is a schematic representation of a preferred embodiment of the invention. Fig. 1 shows three linked DNA cassettes or elements. The three components shown, which may be operably linked but need not be, are a TRRDC, (**T**etracycline **R**esistance (or protection) and **R**epressor **DNA** **C**assette); a TCE (**T**etracycline-**C**ontrollable **E**lement); and RG (**R**eporter **G**ene), together the components, which need not be linked are called the RDC (**R**egulatory **DNA** **C**assette). Arrowheads represent transcription start sites and the direction of transcription. The two octagons represent transcription terminators. Boxes represent coding regions for the genes, the arrows show the direction of transcription of these genes. The open circles represent *tetO* sequences, where tetracycline-repressor protein binds in the absence of tetracycline. Vertical bars represent restriction endonuclease cleavage sites. The region between the cleavage sites between the *tetR* and *BlaZ* coding regions is the TCE region. The *tetM*, *tetR*, TCE and *BlaZ* are described herein. **Fig. 1** shows a particular embodiment of this invention because it shows three transcription promoter systems, the TCE, the TRRDC and the RG combined in a single DNA element where in fact, neither the TRRDC nor the RG must be in the same DNA construct as the TCE.

Figure 2, SEQ. ID. NO. 33, is the nucleotide sequence of the synthetic DNA fragment of the regulatory cassette containing two transcription terminator sequences. The nucleotides in bold letters comprise recognition sequences for the restriction endonuclease indicated above in italics. The dotted arrows indicate the regions of dyad symmetry of the rho-independent terminator sequences where putative stem-loops form followed by a string of T's during transcription.

Figure 3, SEQ. ID. NO. 34, is the nucleotide sequence of the amplified DNA fragment for the element of the regulatory cassette encoding tetracycline resistance gene, the *tetM*. The nucleotides in bold letters comprise recognition sequences for the restriction endonucleases indicated above in italics. The DNA represents the coding strand for the gene, with transcription and translation occurring from top to bottom as shown in this Figure.

Figure 4a, SEQ. ID. NO. 35, is the nucleotide sequence of the amplified DNA sequence for the element. The nucleotides in bold letters comprise recognition sequences for the restriction endonucleases indicated above in italics. The DNA

represents the coding strand for the gene, with transcription and translation occurring from top to bottom as shown in this figure.

Figure 4b, SEQ. ID. NO. 36, is the nucleotide sequence of Figure 4a with additional sequence from the 5' untranslated region of the *tetR* gene. The nucleotides in bold letters comprise recognition sequences for the restriction endonucleases indicated above in italics. The DNA represents the coding strand for the gene, with transcription and translation occurring from top to bottom as shown in this figure.

Figure 5, SEQ. ID. NO. 37, is the nucleotide sequence of the synthetic DNA fragment of the regulatory cassette containing two diverging transcriptional promoters *with tetO* sequences. The nucleotides in bold letters comprise recognition sequences for the restriction endonucleases indicated above in italics. Capitalized nucleotides on both DNA strands represent *tetO* sequences, putative binding sites for the tet repressor protein in the absence of tetracycline. The -35 and -10 regions of the *tet* promoter (P_{tet}) and *xyl* promoter (P_{xyl}) are underlined and overlined, respectively. The capitalized ATG on the bottom strand indicates the start codon of the *tetR* open reading frame.

Figure 6a, (**SEQ. ID. NO. 38**) and **Figure 6b** (**SEQ. ID. NO. 39**) are the nucleotide sequences of alternative amplified DNA elements for the regulatory cassette encoding the reporter gene, *BlaZ*. The nucleotides in bold letters comprise recognition sequences for the restriction endonucleases indicated above in italics. The DNA represents the non-coding strand of the DNA, with transcription and translation going from top to bottom in this figure. **Figure 6a** (**SEQ. ID. NO. 38**) represents the sequence which would be used for constructs where the cassette could be integrated into the chromosome. **Figure 6b** (**SEQ. ID. NO. 39**) represents the sequence which would be used for constructs where the reporter gene is cloned downstream of the target gene.

Figure 7a, SEQ. ID. NO. 40, is the nucleotide sequence of the amplified DNA homologous to *Staphylococcus aureus* chromosomal DNA upstream to the endogenous structural gene for elongation factor Tu (EF-Tu). **Figure 7b, SEQ. ID. NO. 41,** is the nucleotide sequence of the amplified DNA homologous to *Staphylococcus aureus* chromosomal DNA overlapping the 5' end of the structural gene for EF-Tu. The

nucleotides in bold letters comprise recognition sequences for the restriction endonucleases indicated above in italics.

Figure 8a, SEQ. ID. NO. 42, is the nucleotide sequence of the amplified DNA homologous to *Staphylococcus aureus* chromosomal DNA upstream to the endogenous structural gene for *femA*. **Figure 8b, SEQ. ID. NO. 43,** is the nucleotide sequence of the amplified DNA homologous to *Staphylococcus aureus* chromosomal DNA overlapping the 5' end of the structural gene for *femA*. The nucleotides in bold letters comprise recognition sequences for the restriction endonucleases indicated above in italics.

Figure 9a, SEQ. ID. NO. 44, is the nucleotide sequence of the amplified DNA homologous to *Staphylococcus aureus* chromosomal DNA upstream to the endogenous structural gene for *lgt*. **Figure 9b, SEQ. ID. NO. 45,** is the nucleotide sequence of the amplified DNA homologous to *Staphylococcus aureus* chromosomal DNA overlapping the 5' end of the structural gene for *lgt*. The nucleotides in bold letters comprise recognition sequences for the restriction endonucleases indicated above in italics.

Detailed Description of the Invention

Definitions. Throughout this document words and phrases are used that should be known to one skilled in the art. A PhD scientist having experience in the field will know what is described here. The documents incorporated by reference define many terms. In some cases special words, phrases or abbreviations are used that are unique to this document. The meaning of those special or unique words, phrases or abbreviations can be learned either from reading them in context and/or they are decribed immediately below.

C when followed by a number refers to temperature in degrees celsius. The C may be followed by a slash "/" and a number, or the C may be followed by a supercript "^{O}" and a number, e.g. C/37 or C^{O}37.

beta-lactamase or β-lactamase - is a reporter gene and protein, it is further described below.

gene product - means any protein, enzyme, nucleic acid, ribosome components, compounds, even sugar coded by or directly resulting from a protein whose sequence was coded for by the subject gene.

RDC - means a stable **R**egulatory **DNA** **C**assette, it is further described below.

RE - means **R**ecombining **E**lements, it is further described below.

TCE - means a **T**etracycline-**C**ontrollable **E**lement, it is further described below.

TRRDC - means a **T**etracycline **R**esistance (or protection) and **R**epressor **DNA** **C**assette (TRRDC), it is further described below.

RG - means reporter gene, it may also be called a marker gene or enzyme. Sometimes when read in context reporter gene will refer to the reporter protein.

tetO - means **tet**racycline **o**perator sequences, it is further described below.

micron - can be abbreviated with the symbol "u" or "μ."

Tn10 - means means a bacterial transposon that can confer tetracycline resistance in *E. coli* and other enterobacteria, it is further described below.

Here we describe a way to identify which microbial genes are essential for maintaining an infection. We disclose a screen designed to genetically engineer microbial pathogens so that expression of specific genes can be regulated *in vitro* and <u>during host infection</u>. To accomplish this, heterologous DNA sequences are inserted into the bacterial chromosome to disrupt wild type expression of a targeted gene. Expression of the targeted gene is then regulated by inserting a regulatory cassette into the chromosome such that the regulatory cassette controls expression of the targeted gene. Alternately the targeted gene can be cloned and put under the control of a regulatory cassette somewhere else in the chromosome or on an extrachromosomal DNA fragment. This theory can be applied to any gene regulatory system where the gene is regulated and controlled by regulatory elements and where the regulatory elements respond to exogenous influences. Examples exist of regulatory elements controlled or influenced by such things as for example, beta-lactamase, beta-galactoside and nutritional factors such as sugars, (glucose, etc.), amino acids, (tryptophan, etc,) and chemical elements, (iron, etc.).

Applicant's incorporate the definitions, theories and descriptions of PCT application PCT/US96/07937, International Publication Number WO 96/40979, published 19 December 1996 (19.12.96), in part, into this document by reference. Recombinant sequences and the examples disclosed in PCT/US96/07937 are NOT incorporated into this document.

US 5,464,758, published 7 Nov. 1995 is incorporated in part here, the general definitions, theories, principles, concepts, general information about the tet operator (tetO) sequences is incorporated into this document by reference but the sequences disclosed in US 5,464,758 are NOT incorporated into this document.

Here we specifically describe gene regulatory elements which respond to the presence or absence of tetracycline. Tetracycline is thus used to regulate the expression of the targeted genes. Because tetracycline is not normally present in animals, a tetracycline-regulated microbial gene can be controlled *in vivo* by adding or removing tetracycline from the infected animals' diet.

This invention describes a method for evaluating microbial gene products as targets for antimicrobial agents. Antibiotics work by targeting a microbial process essential for survival of the microbe in the infected host. By genetically engineering microbes so that genes can be shut off while the microbes are infecting a mammal it allows us to mimic the effect of a compound that inhibits a process where the gene product is involved. If the gene product is required by the microbe for survival in the host, turning off the gene is comparable to treating the infection by administering antibiotics that target any process in which that gene is involved. In this way, we can test the effect of inhibiting these steps without having to first screen for specific chemical inhibitors.

PCT publication, WO 96/40979, assigned to Microcide Pharmaceuticals, Inc. suggests it might be possible to regulate the genes of a microbe during an infection, but the document does not describe how this could be done. The description provided in this document now describes a method for genetically engineering a microbe so that a specific gene of interest in the microbe can be regulated while the microbe is infecting a mammal. This genetically engineered system for regulating genes of interest is controlled by the presence or absence of tetracycline. In this invention, a mammal could be infected with the genetically engineered microbe while feeding the mammal tetracycline. The system is designed such that the gene is expressed in the presence of tetracycline. Once the infection is established, tetracycline is removed from the diet, turning off expression of the gene. If the target is a gene or gene product required for the infection, removing the tetracycline and turning off the gene should clear the infection from the mammal.

Genetic engineering of the microorganism requires the incorporation of a TCE into the microbe. TCE means a **T**etracycline-**C**ontrollable **E**lement, and it is more fully described below.

The TCE can be made into part of a defined DNA unit or DNA cassette, which can contain about 5 or 6 different elements. These elements are all shown as part of **Figure 1**. Those elements can include: a) 1 to several transcription terminators; b) the structural gene *tetM*, c) the structural gene *tetR*; d) 1 to several promoters; e) a reporter element or reporter gene, which is here exemplified by the structural gene for *BlaZ*. f) These different elements have restriction sites which allow compatible ends and this allows for ligation of the different elements into the DNA cassette. The entire DNA cassette shown in **Fig. 1** is called the **R**egulatory **DNA** **C**assette or the **RDC**.

Note, the structural gene *tetM*, and the structural gene *tetR* do not need to be part of the RDC *per se*, rather they can be on a different plasmid or otherwise inserted into the microbe in a manner where they are expressed by the microbe, but they do not need to be controlled by the promoters in the RDC. The structural gene *tetM*, the structural gene *tetR* and a promoter sequence are referred to here as the tetracycline resistance (or protection) and repressor DNA cassette (TRRDC). As is used in this document, the tetracycline repressor gene refers to the structural gene *tetR* and its associated protein, the tetracycline repressor protein refers to the structural protein TetR. As is used in this document, the tetracycline resistance gene refers to the structural gene *tetM* and its associated protein, the tetracycline resistance protein refers to the structural protein, TetM. The function, purpose and design of the *tetR* and *tetM* genes and gene products are more fully discussed below. The components of the TRRDC are shown in **Fig. 1**. The three elements, the TRRDC, the TCE and the Reporter Gene (RG), are all shown in **Fig. 1**.

The transcription terminators also are not required in the TRRDC but they may be in the TRRDC, as is shown in **Fig. 1**. The reporter gene, RG, can be any gene which expresses a gene product which can be quantitatively assayed. Here we have found the *BlaZ* gene makes a preferred reporter gene. Thus, **Fig. 1** is shown to be a particular embodiment of this invention. **Fig. 1** shows two transcription promoter systems in a single DNA element or cassette where in fact they do not need to be

combined in this manner. What is required is that the target gene and the reporter gene both be controlled by the same promoter system, and this system is regulated by tetracycline. The structural genes for the structural gene *tetM*, and the structural gene *tetR* can be controlled by a promotor or promotors from any source that functions in the microbe, such as a separate plasmid.

The key component of the RDC, is the TCE (tetracycline-controllable element) which is a gene regulatory system that controls the expression of the target gene, or gene product. The target, or gene product being evaluated as a target for antimicrobial treatment is controlled by a transcription promoter that in turn is regulated by a tetracycline repressor protein encoded by *tetR*. In the absence of tetracycline, the *tetR*-encoded protein binds tetracycline operator sequences (*tetO*) around the transcription promoter, reducing or preventing transcription from the promoter. In the presence of tetracycline, the *tetR*-encoded protein binds tetracycline, preventing binding to the *tetO* sequences, allowing transcription from the promoter. (TCE) has the promoter sequences allowing for transcription of the target gene and includes *tetO* sequences. In this example we have included the *tetR* gene in the RDC, but it could be incorporated into the microbe as a separate component, either as a chromsomal insertion or on a plasmid vector.

The tetracycline-controllable element (TCE) system in the example shown here is based on regulatory elements of a tetracycline-resistance operon. Tn10 is a transposon with a tetracycline-regulatory system. Tn10 is described in Hillen & Wissmann, "Topics in Molecular and Structural Biology," in *Protein-Nucleic Acid Interaction*, Saeger and Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp. 143-162), incorporated by reference into this document. Transcription of resistance-mediating genes within Tn10 is negatively regulated by a tetracycline repressor (TetR). In the presence of tetracycline or a tetracycline analogue, TetR does not bind to its operators located within the promoter region of the operon, allowing transcription. Promoters operably fused to tetracycline operator (*tetO*) sequences are virtually silent in the presence of TetR and low concentrations of tetracycline.

The specificity of the TetR for its operator sequence (Hillen & Wissmann, "Topics in Molecular and Structural Biology," in *Protein-Nucleic Acid Interaction*, Saeger & Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp. 143-162) as well as

the high affinity of tetracycline for TetR (Takahashi et al., *J. Mol. Biol.* 187:341-348 (1986)) and the well-studied chemical and physiological properties of tetracyclines constitute a basis for an inducible expression system in prokaryotic cells.

The present invention also relates to a second polynucleotide molecule coding for a protein, wherein said polynucleotide is operably linked to a minimal promoter operatively linked to at least one tet operator (*tetO*) sequence. The *tetO* sequence may be obtained, for example according to Hillen & Wissmann, "Topics in Molecular and Structural Biology," in *Protein-Nucleic Acid Interaction*, Saeger & Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp. 143-162, the contents of which are fully incorporated by reference herein. Other *tetO* sequences which may be used in the practice of the invention may be obtained from the references given in the following: Waters et al., *Nucl. Acids Res.* 11:6089-6105 (1983); Postle et al., *Nucl. Acids Res.* 12:4849-4863 (1984); Unger et al., *Gene* 31:103-108 (1984); Unger et al., *Nucl. Acids Res.* 12:7693-7703 (1984); Tovar et al., *Mol. Gen. Genet.* 215:76-80 (1988); for comparison and overview see Hillen & Wissmann in *Protein-Nucleic Acid Interaction*, Saeger & Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp. 143-162 and can also be utilized for the expression system described. All references in this paragraph incorporated by reference into this document.

To prevent killing of the microbe by the tetracycline used to regulate the system, a gene encoding a protein that confers tetracycline resistance is also added to the construct. Tetracyline functions as an antibiotic by interferring with an elongation factor required for protein synthesis. Some genes conferring tetracycline resistance express a gene product that would effect the tetracycline levels in the cell, either by pumping tetracycline out of the cells, or by chemically altering the tetracycline. Because tetracycline is needed to regulate the TCE, it is important to use a tetracycline resistance gene that does not alter the tetracycline levels in the microbe. Specifically, the *tetM* gene, a tetracycline resistance gene, was chosen to provide tetracycline protection to the microbe. The *tetM* gene encodes a protein believed to be an alternative ribosomal elongation factor that can function in the presence of tetracycline. Here we describe adding the *tetM* gene to make the RDC, but it too could be added seperately to the microbe, by insertion into the chromsome or on a plasmid vector.

In addition, a reporter gene may be added to the construct that allows for an easy way to measure the amounts of protein expressed from a gene under control of the RDC. Alternatively the reporter gene may be present in the microbe. In our case, the gene *BlaZ*, encoding β-lactamase is used as the reporter gene. This gene allows for selection of expression in that it confers resistance to β-lactams. That is, organisms expressing this gene can be selected by their survival in the presence of β-lactams. Furthermore, the levels of β-lactamase can be quantitatively assayed by a simple colorimetric assay. By following the levels of β-lactamase activity in the presence or absence of tetracycline, we can measure the sensitivity of the TCE, using this to select optimized TCE sequences.

The TCE must then be linked to the target genes in the microbe. This can be accomplished in several ways. Here two promenent methods will be discussed as Option I and Option II. Other options should be apparent to one ordinarily skilled in the art.

Option I. The TCE alone; the TCE ligated to tetR, tetM and BlaZ ; or the full RDC, can be inserted into the chromosome. Recombining elements (RE) flanking the inserted DNA should be designed to have enough sequence identity with the host chromosomal DNA to allow homologous recombination into the chromosome. The RE sequences are designed to target insertion so that the cassette is between the target gene and it's endogenous transcription promoter sequences. In this way, the natural controlling seqeunces are removed from the target gene, and the target gene expression is controlled by the TCE as inserted or the TCE as part of the RDC.

Option II. Another method for linking the target genes to the TCE involves introduction of the target gene between the TCE (either alone; or ligated to tetR, tetM and BlaZ; or as part of the RDC) and the reporter gene or just after the reporter gene on a plasmid vector in the microbe. In this Option II method, a microbe is used which has the wildtype target gene from the chromosome inactivated. The target gene is then ligated into the TCE containing DNA fragment and inserted into a suitable plasmid vector for stable transformation of the microbe.

The genetically engineered microbe is then used to infect a sample of mammals such as mice. For example, two groups of mice, say Group A mice and

Group B mice, are all treated with tetracycline (possibly by adding tetracycline to their drinking water) while being infected with the microbe. In both groups of mice, the gene in the infecting microbe should be on and producing functional product because the microbe is exposed to tetracycline being fed to the animals. Tetracycline is then removed from the water of the Group B mice. The Group A and Group B mice are then compared over time. Because the Group A mice are still exposed to tetracycline, the target gene in the microbe should be on and functioning in Group A infections. But in the Group B mice, expression of the target gene in the infecting microbe should be reduced, or even turned off, once the tetracycline is removed. If the Group A mice, the mice with microbes having a functioning gene, continue to show signs of infection and continue to get sick and possibly even die, while at the same time the Group B mice, infected with microbes where the gene is turned off, and thus producing less gene product, may be able to recover from the infection, or they may show signs of improvement, or if they at least don't die, then one knows that the controlled gene or gene product is important for the microbe to sustain the infection and should be selected as an antimicrobial target. This type of difference would be considered a significant difference. Any significant difference would also be considered a meaningful difference between the two groups of animals. Significance can also be quantified with well known statistical tests. A meaningful difference could be determined by one ordinarily skilled in the art of evaluating microbial infections.

If both Group A and B mice continue to get sick or continue to suffer from the microbial infection, after tetracycline is removed from the diet of Group B mice, that indicates the gene is probably not a good target for further antimicrobial research, because inhibiting the protein or gene product probably will not cure the infection in a mammal anyway.

This is just one example of how the system may be used, obvious variations of the above example should be apparent to one skilled in the art. The invention being described above, the authors would now like to provide a few preferred embodiments of the invention.

Preferred Embodiments

A preferred embodiment of the invention relates to an isolated DNA molecule, or DNA cassette, for integrating a heterologous polynucleotide sequence at a pre-determined location in a microbial chromosome to operably control an endogenous prokaryotic gene or as an extrachromasomal element cloned such that it operably controls a functional copy of the targeted gene, the DNA molecule comprising a tetracycline controllable element (TCE) where the TCE comprises a tetracycline-controllable prokaryotic transcription promoter. For integration into the microbial chromosome, the TCE polynucleotide sequence is flanked at its 5' end, and optionally at the 3' end, by a recombining elements (RE), where the RE comprises additional polynucleotide sequences of sufficient length for homologous recombination between the isolated DNA molecule and the microbial chromosome.

In a preferred embodiment, the isolated DNA molecule referred to above further comprises a polynucleotide sequence, which encodes a reporter gene, that is operably linked to the TCE. The reporter gene can be a fluorescent marker, an enzyme such as beta-galactosidase, a protease, here the preferred reporter gene is beta-lactamase.

In an alternative preferred embodiment, the isolated DNA molecule referred to above further comprises at least one transcription terminator polynucleotide sequence positioned between the RE and the TCE.

In yet another preferred embodiment, the isolated DNA molecule referred to above further comprises a polynucleotide sequence, which encodes a prokaryotic tetracycline resistance protein, operably linked to a transcription promoter polynucleotide sequence positioned between the RE and the TCE. Preferably, the tetracycline resistance protein is derived from the *Staphylococcus aureus tetM* gene.

In another preferred embodiment, the isolated DNA molecule referred to above further comprises a polynucleotide sequence, which encodes a prokaryotic tetracycline repressor protein, operably linked to a tetracycline-controllable prokaryotic transcription promoter polynucleotide sequence positioned between the RE and the TCE. Preferably, the tetracycline repressor is derived from the transposon Tn10, see Postle, K., Nguyen, T. T., and Bertrans, K. P., 1984, *Nucleic Acids Research* 12:4849-4863, incorporated into this document by reference.

In an alternative preferred embodiment, the isolated DNA molecule referred to above is a recombinant vector in a form suitable for transformation of a host cell. Another preferred embodiment comprises a host cell transformed with this recombinant vector.

Another preferred embodiment comprises a microbial host cell comprising the DNA molecule referred to above wherein the DNA molecule is integrated at a pre-determined location in the host cell chromosome.

An alternative preferred embodiment of the invention relates to an isolated DNA molecule for integrating a polynucleotide sequence including tetracycline-controllable elements (TCE) at a pre-determined location in a target DNA molecule, the isolated DNA molecule comprising the following DNA elements fused in sequence: a first transcription terminator polynucleotide sequence; a second transcription terminator polynucleotide sequence; a polynucleotide sequence encoding a prokaryotic tetracycline resistance protein; a polynucleotide sequence encoding a prokaryotic repressor protein; a first tetracycline-controllable transcription promoter polynucleotide sequence; a second tetracycline-controllable transcription promoter polynucleotide sequence; and a polynucleotide sequence encoding a reporter protein; the isolated DNA molecule comprising a polynucleotide sequence including the TCE flanked at the end opposite the polynucleotide sequence encoding the reporter protein by additional polynucleotide sequences of sufficient length for homologous recombination between the isolated DNA molecule and the target DNA molecule at a pre-determined location. In a preferred embodiment, a recombinant vector comprising this isolated DNA molecule is in a form suitable for transformation of a host cell. In a further preferred embodiment, this isolated DNA molecule is integrated at a pre-determined location in a microbial host cell chromosome.

In an alternative preferred embodiment, the DNA relates to a recombinant vector suitable for the transformation of the microbial pathogen containing the following items: a polynucleotide sequence encoding a prokaryotic tetracycline resistance protein; a polynucleotide sequence encoding a prokaryotic repressor protein; a first tetracycline-controllable transcription promoter polynucleotide sequence; with the following in sequence: a second tetracycline-controllable transcription promoter polynucleotide sequence; an isolated DNA molecule

comprising a polynucleotide sequence encoding the targeted gene; and a polynucleotide sequence encoding a reporter protein.

A preferred embodiment is a DNA cassette as shown in **Fig. 1** and as the components of **Fig. 1** are described in this document.

The above descriptions should completely describe the invention and the examples below, both synthesis examples and working models are provided to illustrate but not limit the above descriptions of the invention.

EXAMPLES

Materials and Methods

Construction of Tetracycline-responsive DNA regulatory cassette:

A DNA cassette is constructed for introduction into *S. aureus* either by homologous recombination into the *S. aureus* chromosome at a specific site by Campbell-type recombination, see Campbell, A., 1962, *Advan. Genet.* 11, 45-101, incorporated into this document by reference, or on an autonomously replicating plasmid. For chromosomal integration, this DNA contains a region at one or both ends homologous to regions of the *S. aureus* chromosomal DNA. The rest of the construct contains a recombinant DNA cassette as illustrated in **Fig. 1.** On an autonomously regulated plasmid, the recombinant DNA cassette in Figure 1 would contain DNA encoding a *S. aureus* gene.

The first element of this cassette contains two transcription terminators, which are designed to prevent transcriptional read-through from the chromosomal DNA into this insert as well as transcriptional read-through from the cassette into the chromosome. These are followed by a *S. aureus* gene conferring resistance to tetracycline, *tetM*. This gene was chosen because the mechanism of resistance does not appear to change the structure or concentration of tetracycline in the cell, rather it appears to provide an alternative elongation factor which is resistant to the tetracycline in translation, see Nesin, M., Svec, P., Lupski, J. R., Godson, G. N., Kreisworth, B., Kornblum, J. and Projan, S. J., Antimicrob. Agents Chemother., 1990, 34:2273-2276, incorporated into this document by reference. This gene is transcribed from left to right as shown in **Figure 1.** Alternatively, *tetM* could be incorporated somewhere else in the chromosome of *S. aureus* to provide a background strain useful for a number of targeted gene tests. The gene encoding *E. coli* tet repressor, *tetR* , see

Postle, K, Nguyen, T. T., and Bertrand, K. P., *Nuc. Acids Res.*, 1984, 12:4849-4863, incorporated into this document by reference, is transcribed as an operon with *tetM* from an adjacent promoter on the region containing two diverging promoters (P_{tet} and P_{xyl}) and two tetracycline operator sequences (*tetO*). The tet repressor protein binds *tetO* sequences in the absence of tetracycline, preventing transcription from P_{xyl}. In the presence of tetracycline, tet repressor binds tetracycline and not *tetO* sequences, allowing transcription from P_{xyl}. The strong *B. subtilis* promoter, P_{xyl}, signals initiation of transcription to the right as drawn **in Figure 1**, allowing transcription of *S. aureus BlaZ* encoding beta-lactamase, an assayable marker gene which confers resistance to ampicillin, see Wang, P. Z. and Novick, R. P., 1987, *J. Bacteriol.*, 169:1763-1766, incorporated into this document by reference. When this DNA is inserted into the chromosome, the gene being tested as a target should be transcribed in an operon with *BlaZ*, and have similar transcriptional regulation. When the DNA is contained on an autonomously regulated plasmid, the DNA encoding the target gene would be inserted next to *BlaZ* so that the target gene and *BlaZ* should be transcribed in a single operon and have similar regulation.

The following paragraphs describe how each of the DNA cassette elements are made. For totally synthetic elements (1 and 4), DNA oligonucleotides are designed to leave overhanging nucleotides at both ends that resemble the sticky ends left by digestion with restriction endonucleases. For elements amplified by PCR, oligonucleotides are designed to incorporate unique recognition sites for restriction endonucleases on both ends. These restriction sites simplify ligations with each other and with restriction enzyme digested plasmids. Oligonucleotides were synthesized by Genosys Biotechnologies, Inc., The Woodlands, TX.

DNA ligations are performed in T4-DNA ligation buffer (50 mM Tris HCl, pH 7.6, 10 mM MgCl2, 10 mM dithiothreitol, 50 ug/ml bovine serum albumin) with T4-DNA ligase (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 14/C overnight. In general, PCR reactions are carried out in 50 ul reaction volumes using Taq polymerase and reaction buffer from Perkin-Elmer (produced by Roche Molecular

Systems, Inc., Branchburg, NJ). PCR reactions contained 40 uM each of dATP, dCTP, dGTP, and dTTP; 200 nM of each primer; and 1-100 ng chromosomal DNA or plasmid DNA. PCR reactions are heated at 95/C for 5 minutes to denature template, followed by 30 cycles of heating at 95/C for 1 minute, primer annealing at 50/C for 1 minute and elongation at 72/C for 1 minute.

Construction of Element 1: Terminators.

The sequence for the bidirectional terminators are derived from published *S. aureus* transcriptional terminators for *sarA* (Bayer, et al., *J. Bacteriol.*, 1996, 178:4563-4570) and for *pcrB* (Iordenescu, S., *Mol. Gen. Genet.*, 1993, 241:185-192). This element was constructed from four oligonucleotides listed in Table I as CLQ459, CLQ460, CLQ461 and CLQ 462. Before annealing, 5 pmoles of CLQ460 and CLQ461 were treated at 37/C for 30 minutes with T4-polynucleotide kinase (New England Biolabs, Beverly, MA) in 2 mM ATP, 100 mM Tris HCl, pH 7.6, 200 mM spermidine, 10 mM DTT. The reaction was stopped by heating to 85/C for 20 minutes. The kinased CLQ460 and CLQ461 were then mixed with equimolar amounts of CLQ462 and CLQ463, respectively, before heating to 90/C for 5 minutes, followed by cooling to room temperature over 30 minutes. The two pairs of annealed primers were then mixed in equimolar amounts, heated to 50/C for 5 minutes and allowed to cool to room temperature over 30 minutes. The cassette was ligated as described above before ligating with pUC18 plasmid which had been digested with restriction enzymes KpnI and XmaI. **Figure 2** shows the polynucleotide sequence of this DNA fragment.

Construction of Element 2: Tetracycline Resistance.

The structural gene of *S. aureus tetM* (Genbank accession number M21136) was amplified by PCR as described above, using primers CLQ463 and CLQ464 listed in Table I. These primers add unique recognition sites for the restriction enzymes BamHI and XmaI, respectively. The template for amplification was provided by Serban Iordenescu (Public Health Research Institute, NY), plasmid pRN6880, and is derived from the plasmids published by Nesin, M., Svec, P., Lupski, J. R., Godson, G. N., Kreisworth, B., Kornblum, J. and Projan, S. J., *Antimicrob. Agents Chemother.*, 1990, 34:2273-2276. **Figure 3** shows the polynucleotide sequence of this DNA fragment.

Construction of Element 3: Tetracycline Repressor.

E. coli tetR (Genbank accession number J1830) was amplified by PCR using primers CLQ465 and CLQ467 or CLQ466 and CLQ467 from an *E. coli* strain carrying Tn10 (Hillen, W. and Schollmeier, K, *Nuc. Acids Res.*, 1983, 11:525-539). Primers CLQ465 and CLQ467 incorporate unique recognition sites for the restriction endonucleases SpeI and BamHI, respectively and include the wildtype promoter sequence for this gene. When primer CLQ466 is paired with CLQ467, it amplifies a shorter region of *tetR*, starting near the XbaI restriction enzyme recognition site found near the start codon of the gene. This shorter construct allows for the cloning of non-wildtype leader and promoter sequences to control this gene. PCR reactions were carried out using whole cells after heating the reaction mixture to 95/C for 5 minutes and cycling 35 times through three successive steps of 95/C for 1 minute, 45/C for 1 minute and 72/C for 1 minute. The PCR product was cloned using the pT7-Blue-T vector kit (Novagen, Madison, WI) according to the manufacturer's instructions. **Figures 4a and 4b** show the polynucleotide sequences of these DNA fragments.

Construction of Element 4: Transcriptional Promoters.

The synthetic promoter region contains two diverging transcription initiation signals and is derived from the one described by Geissendorfer and Hillen (*Appl. Microbiol. Biotechnol.*, 1990, 33:657-663). It was constructed from oligonucleotides shown in Table I as CLQ468, CLQ469, CLQ 470, CLQ471, CLQ472 and CLQ480. Conditions for kinasing, annealing and ligating these primers were as described for construction of Element 1. Oligonucleotides CLQ469, CLQ470, CLQ471, and CLQ472 were kinased before annealing CLQ469 with CLQ468, CLQ470 with CLQ471 and CLQ472 with CLQ480. After this annealing equimolar amounts of each pair was annealed with the other two pairs before ligation to each other and with pUC18 digested with restriction enzymes XbaI and PstI. When all 6 oligonucleotides were used to construct the promoter cassette, the *tetR* gene amplified with primers CLQ466 and CLQ467 was ligated to it and *tetR* will be transcribed from non-wild-type leader and promoter sequences. Alternatively, when the wildtype promoter and leader sequence from the *tetR* gene was included on the PCR fragment (using PCR primers CLQ465 and CLQ467 for amplification), the synthetic promoter element constructed with only oligonucleotides CLQ470, CLQ471, CLQ472 and

CLQ473 was ligated to it. **Figure 5** shows the polynucleotide sequence of this DNA fragment.

Construction of Element 5: Reporter Gene.

The *S. aureus BlaZ* gene (Genbank accession number M15526), encoding beta-lactamase, was PCR amplified from plasmid pSA3800 (Novick, R. et al., *Cell*, 1989, 59, 395-404) using oligonucleotides CLQ486 and CLQ475 (element 5a) or CLQ486 and CLQ500 (element 5b) from Table 1. CLQ486 incorporates a unique recognition sequence for the restriction endonuclease Pstl. CLQ475 includes unique recognition sites for the restriction endonucleases SphI and EcoRI. CLQ500 includes unique recognition sites for the restriction endonuclease PmeI. The PCR products were cloned using the pT7-Blue-T vector kit (Novagen, Madison, WI). Figures 6a and 6b show the polynucleotide sequence of these DNA fragments.

After all the PCR and synthetic DNA elements are assembled into a single cassette, the DNA cassette is ligated in a *S. aureus* plasmid. For those constructs designed to integrate into the chromosome, the cassette is also ligated to insertion-directing sequences made of homologous chromosomal DNA. The plasmid is passaged through *S. aureus* RN4220, see Peng, H.-L., Novick, R. P., Kreiswirth, B., Kornblum, J. and Schlievert, P., 1988, *J. Bacteriol.* 170, 4365-4372, incorporated into this document by reference, a restriction minus, modification positive strain. Plasmid DNA purified from RN4220 is modified by native *S. aureus* DNA modification enzymes and is more readily transformed into pathogenic *S. aureus* strains that have wild-type DNA restriction systems, see Iordenescu, S. and Surdeanu, M., 1976, *J. Gen. Microbiol.* 96, 277-281, incorporated into this document by reference. Insert DNA released by EcoRI restriction enzyme digestions is purified and circularized. This DNA is transformed into a pathogenic *S. aureus* strain, selecting for tetracycline resistance. Because the insert DNA does not have an origin of replication, it should not be maintained as an autonomous plasmid, and growth on tetracycline selects for recombinants where the cassette has been inserted into the chromosome. Southern blots or PCR analysis are used to verify that the desired recombination event has occurred.

For regulation of a target gene on an autonomously replicating plasmid, the DNA cassette ligated into a suitable plasmid vector is passaged through *S. aureus*

RN4220 for modification and then directly transformed intact into another *S. aureus* strain. This strain may be derived from a pathogenic strain but genetically engineered so that expression of the endogenous copy of the target gene is altered from the pathogenic parent.

Alternatively, the genes encoding tetracycline resistance and the tetracycline repressor with a promoter sequence can be recombined separately into another region of the *S. aureus* chromosome. These genes do not need to be adjacent to the other DNA elements of the regulatory cassette. The DNA elements containing the transcription terminators, tetracycline regulated promoter and the β-lactamase reporter gene can still be constructed so that they recombine between the target gene and its transcription regulatory elements on the wild-type chromosome.

The beta-lactamase reporter gene allows for measurement of transcriptional read-through at different tetracycline concentrations. If the tetracycline regulation works as expected in this system, the cells should make less beta-lactamase and the test gene at lower tetracycline concentrations. Ideally, no detectable levels of β-lactamase or the test gene would be found in the absence of tetracycline. If transcription of the test gene can be turned off in this way and the gene being tested is an essential gene, the cells should not survive in the absence of tetracycline. If the gene is not essential and appears to be regulated by tetracycline in this system, its potential as an antimicrobial target will be tested in an animal infection model. Animal infections are established with this genetically engineered bacteria while feeding tetracycline to the animals. We will look for clearing of the infection when tetracycline is removed from the infected animals' diet.

Example 1

In the first example, the validity of this approach is tested by controlling the regulation of a gene essential for *S. aureus* growth on minimal media lacking exogenous tryptophan: *trpD*, a gene encoding an enzyme of the tryptophan biosynthetic pathway. The strtuctural gene for *trpD* from *S. aureus* chromosomal DNA was PCR amplified with specific primers adding polynucleotide sequences for recognition by PstI endonuclease to each end. This PCR construct is ligated between the promoter (element 4a or 4b) and the BlaZ structural gene (element 5b) so that it will be transcribed from left to right as drawn in Figure 1. When cells are

transformed with this construct, the *trpD* gene should be transcribed from the P_{xyl} promoter and transformants can be selected for by growth on tetracycline. This example serves as a positive control for the regulatory system. If the regulatory elements function as predicted, the presence of tetracycline will allow transcription of the beta-lactamase marker gene as well as *trpD*, and the cells will grow on media with or with out ampicillin and with or without tryptophan. In the absence of tetracycline, the tet repressor should bind the promoter, decreasing transcription of beta-lactamase and *trpD*. In this case, the cells would not be expected to survive in the absence of ampicillin or tryptophan. If they do survive, levels of beta-lactamase produced by these cells can be measured at different tetracycline concentrations to determine the level of repression achieved with the tet repressor. As long as there is some repression, this control can be tested in the animal infection to see if an infection established by these cells in the presence of tetracycline can persist in the absence of tetracycline. This is an indicator for how sensitive the system will be in testing target genes.

Example 2

In the second example, the validity of integrating the cassette into the chromosome is tested by controlling the regulation of a gene assumed to be essential for *S. aureus* growth: the gene encoding elongation factor Tu (EF-Tu). EF-Tu is required for protein translation and is a proven target for antibiotics (Selva, E., Montanini, N., Stella, S., Soffietini, A., Gastaksi, L. and Denaro, M., 1997, *J. Antibiot. Tokyo* 50, 22-26), incorporated by reference. Primers CLQ455 and CLQ456 from **Table 1** were used to PCR amplify one 320 base pair fragment from *S. aureus* chromosomal DNA corresponding to a region of DNA just upstream from the EF-Tu structural gene and including the 3' end of the structural gene for elongation factor G (**Figure 7a**). A second fragment, PCR amplified using primers CLQ505 and CLQ506 from **Table 1,** corresponds to a region overlapping the 5' end of the EF-Tu structural gene (**Figure 7b**). The insertional DNA cassette was constructed by ligating these fragments to element 1 and element 5a, respectively. When this DNA fragment is used to transform *S. aureus* cells, the fragments direct recombination of the insert into the chromosome about 20 bp before the putative ribosome binding site for the

EF-Tu gene in the *S. aureus* chromosome. Insertion of the DNA fragment in the chromosome is selected by growth on tetracycline and ampicillin. Recombination into the desired site can be confirmed by Southern Blot or PCR analysis of chromosomal DNA. This example serves as a positive control for the regulatory system. If the regulatory elements function as predicted, the presence of tetracycline will allow transcription of the beta-lactamase marker gene as well as EF-Tu, and the cells will grow on media with or with out ampicillin. In the absence of tetracycline, the tet repressor should bind the promoter, preventing transcription of beta-lactamase and EF-Tu. In this case, the cells would not be expected to survive in the presence or absence of ampicillin because EF-Tu is expected to be essential. If they do survive, levels of beta-lactamase produced by these cells can be measured at different tetracycline concentrations to determine the level of repression achieved with the tet repressor. As long as there is some repression, this control can be tested in the animal infection to see if an infection established by these cells in the presence of tetracycline can persist in the absence of tetracycline. This is an indicator for how sensitive the system will be in testing target genes.

Example 3.

In the third example, the DNA cassette is constructed to allow testing of the *S. aureus femA* gene (Genbank accession number M23918). Elements 1, 2, 3, 4 and 5 are the same as the elements in Example 2. These elements were fused to two pieces of DNA corresponding to *S. aureus* chromosomal DNA around the *femA* structural gene. This gene has been identified as a virulence factor: insertional inactivations of the gene reduce the virulence of a *S. aureus* pathogen (Mei-JM; Nourbakhsh-F; Ford-CW; Holden-DW, Mol-Microbiol. 1997 Oct; 26(2): 399-407.). Primers CLQ451 and CLQ452 from **Table 1** were used to amplify one 369 base pair fragment of *S. aureus* chromosomal DNA just upstream from the *femA* structural gene and including the 3' end of *trpA* (**Figure 8a**). Primers CLQ501 and CLQ502 were used to amplify a seond fragment of *S. aureus* chromosomal DNA overlapping the 5' end of the *femA* structural gene (**Figure 8b**). Ligation of the first fragment to element 1 in the insertional DNA cassette and the second fragment to element 5a directs recombination of the insert into the chromosome about 25 bp before the putative ribosome binding site of *femA* in the *S. aureus* chromosome when cells are

transformed with this construct. Again, insertion of the DNA fragment in the chromosome is selected by growth on tetracycline and ampicillin. Recombination into the desired site is confirmed by Southern Blot or PCR analysis of genomic DNA isolated from the recombinant cells. Variation in repression of beta-lactamase expression in the presence or absence of tetracycline is expected to be similar for that seen in Example 2. However, *femA* is reportedly not an essential gene for growth of the cells *in vitro* (Strander, A. M., Ehlert, K, Labischinski, H., and Berger-Bachi, B., 1997, *J. Bacteriol.* 179:9-16), so these recombinant cells would be expected to grow even if transcription of BlaZ end *femA* is completely repressed in the absence of tetracycline. If *femA* is essential for the establishment of an infection and the absence of tetracycline prevents transcription of *femA*, these cells should not be able to establish an infection unless the animal has tetracycline in it. If *femA* is a good target for antibacterial agents, an infection with these cells established in the presence of tetracycline would be cleared with the subsequent removal of tetracycline.

Example 4

In the fourth example, the DNA cassette is constructed for insertion into the chromosome to allow testing of the *lgt* gene in *S. aureus* (Genbank accession number U35773). Encoding the first enzyme for the post-translational modification in lipoprotein biosynthesis, *lgt* has been shown to be an essential gene in *E. coli* (Gan, K., Sankaran, K, Williams, M. G., Aldea, M., Rudd, K E., Kushner, S. R., and Wu, H. C., 1995, *J. Bacteriol.* 177:1879-1882) and *Salmonella typhimurium* (Gan, K, Gupta, S. D., Sankaran, K, Schmid, M. B. and Wu, H. C., 1993, *J. Biol. Chem.* 268:16544-16550), incorporated by reference. However, the essential nature is believed due to toxic effects of unmodified pro-lipoprotein accumulation in the absence of *lgt* in these bacteria, and it is not yet known if *lgt* is an essential gene in *S. aureus* or if it is a gene required for infection. Primers CLQ453 and CLQ454 from **Table 1** were used to PCR amplify a 450 base pair fragment from *S. aureus* chromosomal DNA corresponding to a region of DNA ending 15 bp upstream from the putative ribosomal binding site for the *lgt* structural gene (**Figure 9a**). Primers CLQ503 and CLQ504 from **Table 1** were used to PCR amplify another fragment of the *S. aureus* chromosome overlapping the 5' end of *lgt* (**Figure 9b**). Ligation of this first fragment to element 1 and the second fragment to element 5a in the insertional

DNA cassette directs recombination of the insert into the chromosome about 25 bp before the putative ribosome binding site of *lgt* in the *S. aureus* chromosome when cells are transformed with this construct. Again, insertion of the DNA fragment in the chromosome is selected by growth on tetracycline and ampicillin. Recombination into the desired site is confirmed by Southern Blot or PCR analysis of chromosomal DNA. Variation in repression of β-lactamase expression in the presence or absence of tetracycline is expected to be similar for that seen in Example 2. If transcription of *BlaZ* is repressed in the absence of tetracycline in this construct, *lgt* should also be repressed and the cells should grow only if *lgt* is not an essential gene. If it is not an essential gene, it can be tested in the animal infection model to determine if shutting off *lgt* transcription clears the infection.

Table 1.

Synthetic oligonucleotides used in PCR amplification or cassette construction.

NAME	SEQUENCE
CLQ451	ACGCACGAGCTCGGTTGCAGATGGCATTGTC (SEQ ID NO:1)
CLQ452	GGGGTACCCCCTCTGCAAATGTCAAA (SEQ ID NO:2)
CLQ453	ACGCACGAGCTCAGATCTTCGCTTGTGCGG (SEQ ID NO:3)
CLQ454	GGGGTACCCGCTGAAGAGATAGCGATTG (SEQ ID NO:4)
CLQ455	ACGCACGAGCTCTTTCAGAAATGTTCGGTTATG (SEQ ID NO:5)
CLQ456	GGGGTACCAAATTTATCTCTCATGATAG (SEQ ID NO:6)
CLQ457	CAGGTACAGCAGTAAGTAAGC (SEQ ID NO:7)
CLQ458	GTCAACGTGAGCGTAGTGACG (SEQ ID NO:8)
CLQ459	CGAAGTTTGATAGATGATACATTCTATTAAACTTCCTTTTTTTATGCTCTGAAA (SEQ ID NO:9)
CLQ460	AAACAATGATTATCTACCTTATTAGTGCAGATAGATAACCATTGTTTATC (SEQ ID NO:10)
CLQ461	AGCATAAAAAAAGGAAGTTTAATAGAATGTATCATCTATCAAACTTCGGTAC (SEQ ID NO:11)
CLQ462	CCGGGATAAACAATGGTTATCTATCTGCACTAATAAGGTAGATAATCATTGTTTTTTCAG (SEQ ID NO:12)
CLQ463	CGGGATCCAATGGAGGAAAATCACATG (SEQ ID NO:13)
CLQ464	TCCCCCCGGGTAGGACACAATATCCACTTGTAG (SEQ ID NO:14)
CLQ465	GACTAGTTTGACAAATAACTCTATCAATGATAGAGTGTC (SEQ ID NO:15)
CLQ466	TAATGATGTCTAGATTAGATAAAAGT (SEQ ID NO:16)
CLQ467	CGGGATCCTTAAGACCCACTTTCACATTT (SEQ ID NO:17)
CLQ468	CTAGACATCATTAATTCCTCCTTTTTGTTGACACTCTATCATTGATAGAGTTATTTGTCAAA (SEQ ID NO:18)
CLQ469	CTAGTTTGACAAATAACTCTATCAATGATAGTGTCAACAAAAAGGAGGAATTAATGATGT (SEQ ID NO:19)
CLQ470	CTAGTTTTTTATTTGTCGAGTTCATGAAAAACTAAAAAAAATTGAC (SEQ ID NO:20)
CLQ471	TTTTTTTTAGTTTTTCATGAACTCGACAAATAAAAAA (SEQ ID NO:21)
CLQ472	ACTCTATCATTGATAGAGTATAATTAAAATAAAAAAGCTGCA (SEQ ID NO:22)

CLQ475	ACATACGCATGCGAATTCTTAAAATTCCTTCATTACACTC (SEQ ID NO:23)
CLQ480	GCTTTTTTATTTTAATTATACTCTATCAATGATAGAGTGTCAA (SEQ ID NO:24)
CLQ486	AACTGCAGTAATATCGGAGGGTTTATTTTG (SEQ ID NO:25)
CLQ500	GTTTAAACTTAAAATTCTTCATTACACTC (SEQ ID NO:26)
CLQ501	GGAATTTTAAGTTTAAACTGCAAATACGGAAATGAAATTAAT (SEQ ID NO:27)
CLQ502	ACATACGCATGCGAATTCAAGTATTGATATGGTAAATATGG (SEQ ID NO:28)
CLQ503	GGAATTTTAAGTTTAAACGAGGAGTAGGTTGAATGGGTA (SEQ ID NO:29)
CLQ504	ACATACGCATGCGAATTCCTTGCGCTAAAATTATAC (SEQ ID NO:30)
CLQ505	GGAATTTTAAGTTTAAACGAATAGGAGAGATTTTATAATGGC (SEQ ID NO:31)
CLQ506	ACATACGCATGCGAATTCACGAGTTTGTGGCATTGGACC (SEQ ID NO:32)

Claims

1. A process to allow the characterization of a microbial gene or genes, here gene,

where said gene encodes a gene product;

where said gene product is a gene target;

where said gene target is important to a microbe's ability to infect or sustain an infection in a mammal, where said microbe is:

genetically altered to become a genetically altered microbe, such that the amount of said gene product produced by said genetically altered microbe is regulated and controlled by a Tetracycline-Controllable Element or TCE;

where said TCE is a gene regulatory system that controls the expression of the target gene or gene product, through its ability to modulate the function of said gene in response to said microbe's exposure to tetracycline, and where said TCE is comprised of a tetracycline-controllable transcription promoter polynucleotide sequence;

where said gene, which may be any gene which encodes a microbial protein, or more generally a microbial gene product, is regulated by said TCE such that said gene produces either greater or lesser amounts of gene product, depending upon whether or not said genetically altered microbe is exposed to tetracycline;

where said mammal is a plurality of at least two or more mammals with said mammals are initially exposed to tetracycline and infected with said genetically altered microbe;

followed by:

the removal of the tetracycline exposed to a portion of said mammals, such that there is at least mammals one or one group of said mammals exposed to tetracycline and another one or group of not exposed to tetracycline;

followed by:

a comparison of the degree of infection, microbe levels, or physiological condition of the mammals exposed to tetracycline, compared to the degree of infection, microbe levels, or physiological condition of mammals not exposed to tetracycline;

followed by:

the identification of said genes, important to a microbe's ability to infect or sustain an infection in a mammal, where the comparison of the mammals exposed to tetracycline compared to the mammals not exposed to tetracycline shows a meaningful difference between the two groups of animals.

2. The process of claim 1, where said TCE is a gene regulatory system that controls the expression of the target gene or gene product, through its ability to modulate the function of said gene in response to said microbe's exposure to tetracycline, and where said TCE is comprised of a tetracycline-controllable transcription promoter polynucleotide sequence, operably linked to a polynucleotide sequence encoding a reporter gene.

3. The process of claim 2, where said tetracycline-controllable transcription promoter polynucleotide sequence, is a prokaryotic transcription promoter.

4. The process of claim 1, where said TCE is a gene regulatory system that controls the expression of the target gene or gene product, through its ability to modulate the function of said gene in response to said microbe's exposure to tetracycline, and where said TCE is comprised of a tetracycline-controllable transcription promoter polynucleotide sequence, operably linked to a polynucleotide sequence encoding a reporter gene (RG) and a target gene (TG).

5. The process of claim 4, where said reporter gene is β-lactamase.

6. The process of claim 1, where said microbe has, in addition to the genetic alterations of claim 1, additional genetic alterations comprising a tetracycline resistance (or protection) and repressor DNA cassette (TRRDC).

7. The process of claim 6, where said TCE is a gene regulatory system that controls the expression of the target gene or gene product, through its ability to modulate the function of said gene in response to said microbe's exposure to tetracycline, and where said TCE is comprised of a tetracycline-controllable

transcription promoter polynucleotide sequence, operably linked to a polynucleotide sequence encoding a reporter gene and a target gene

and where the TCE, the TRRDC, the RG, and the TG are all on the same DNA cassette, which may be referred to as a Regulatory DNA Cassette or RDC.

8. The process of claim 6, where the TRRDC, comprises the structural gene *tetM*, the structural gene *tetR* and where a promoter is operably linked to the TCE.

9. The process of claim 1, where said meaningful difference between the two groups of animals is a mathematically significant difference in the survival rates or the levels of microbes, or levels of infection present in the mammals.

10. The process of claim 9, where said meaningful difference between the two groups of animals is a mathematically significant difference in the survival rates of the groups of animals.

11. The process of claim 10, where said significant difference in the survival rates of the groups of animals shows that animals exposed to tetracycline have poorer health, higher rates of infection, lower survival or higher levels of microbes than animals not exposed to tetracycline.

12. The process of claim 7, where the tetracycline resistent gene of said TRRDC is comprised of sequences from the *Staphylococcus aureus tetM* gene.

13. The process of claim 12, where said tetracycline repressor gene of said TRRDC is derived from the Tn10 transposon.

14. The process of claim 13, where said Tn10 transposon is selected from the sequence of SEQ. ID. NO. 35 and 36.

15. The process of claim 1 where said mammals are mice.

16. The process of claim 1, wherein said recombinant bacterium is a Staphylococcus species.

17. The process of claim 1, wherein said Staphylococcus species is *Staphylococcus aureus*.

18. The process of claim 1, wherein said microbe is a virus.

19. The process of claim 1, wherein said microbe is a lower eukaryote.

20. The process of claim 1, wherein said microbe is a yeast.

21. An isolated DNA molecule for integrating a heterologous polynucleotide sequence at a pre-determined location in a prokaryotic chromosome to operably control an endogenous prokaryotic gene, said DNA molecule comprising recombining element (RE) and a tetracycline controllable element (TCE), said TCE comprising a tetracycline-controllable prokaryotic transcription promoter polynucleotide sequence flanked at its 5' end by said RE, said RE comprising additional polynucleotide sequences of sufficient length for homologous recombination between the isolated DNA molecule and the prokaryotic chromosome.

22. The isolated DNA molecule of claim 21 further comprising a polynucleotide sequence encoding a reporter gene operably linked to said TCE.

23. The isolated DNA molecule of claim 22 wherein said reporter gene is beta-lactamase.

24. The isolated DNA molecule of claim 21 further comprising at least one prokaryotic transcription terminator polynucleotide sequence positioned between the RE and the TCE.

25. The isolated DNA molecule of claim 21 further comprising a polynucleotide sequence encoding a prokaryotic tetracycline resistance protein operably linked to a prokaryotic transcription promoter polynucleotide sequence positioned between the RE and the TCE.

26. The isolated DNA molecule of claim 25 wherein the tetracycline resistance protein is derived from the *Staphylococcus aureus tetM* gene.

27. The isolated DNA molecule of claim 21 further comprising a polynucleotide sequence encoding a prokaryotic tetracycline repressor protein operably linked to a tetracycline-controllable prokaryotic transcription promoter polynucleotide sequence positioned between the RE and the TCE.

28. The isolated DNA molecule of claim 27, wherein the tetracycline repressor is a a *tetR* gene derived from the Tn10 transposon.

29. A recombinant vector comprising the isolated DNA molecule of claim 21 in a form suitable for transformation of a host cell.

30. A host cell comprising the recombinant vector of claim 29.

31. A prokaryotic host cell comprising the DNA molecule of claim 21 wherein the DNA molecule is integrated at a pre-determined location in the host cell chromosome.

32. The isolated DNA molecule of claim 21, wherein said recombining elements are comprised of polynucleotides selected from Sequence ID NO: 40, 41, 42 and 43.

33. The isolated DNA molecule of claim 21, wherein said recombining elements are comprised of polynucleotides selected from Sequence ID NO: 44 and 45.

34. The isolated DNA molecule of claim 21, wherein said tetracycline-controllable element is comprised of polynucleotide Sequence ID NO: 37.

35. The isolated DNA molecule of claim 21, further comprising a polynucleotide sequence encoding a reporter gene operably linked to said tetracycline-controllable element.

36. The isolated DNA molecule of claim 21, wherein said tetracycline-controllable element is comprised of polynucleotide Sequence ID NO: 37.

37. The isolated DNA molecule of claim 21, wherein said reporter gene is beta-lactamase.

38. The isolated DNA molecule of claim 21, wherein said reporter gene is beta-lactamase, selected from SEQ ID NO: 38 and 39.

39. The isolated DNA molecule of claim 21 wherein the tetracycline resistance protein is derived from the *Staphylococcus aureus tetM* gene.

40. The isolated DNA molecule of claim 21 wherein the tetracycline resistance protein is derived from the *Staphylococcus aureus tetM* gene selected from SEQ ID NO: 34.

41. The isolated DNA molecule of claim 21 wherein the tetracycline repressor is a a *tetR* gene derived from the Tn10 transposon and selected from SEQ ID NO: 35 and 36.

42. The isolated DNA molecule of claim 21, further comprising a polynucleotide sequence comprising at least one prokaryotic transcription terminator seqeunce (SEQ ID NO:33) positioned between the tetracycline-controllable element and one recombining element.

43. The isolated DNA molecule of claim 21, further comprising a polynucleotide sequence encoding a prokaryotic tetracycline resistance protein operably linked to a transcription promoter polynucleotide sequence.

44. The isolated DNA molecule of claim 21, further comprising a polynucleotide sequence encoding a tetracycline repressor protein operably linked to a transcription promoter polynucleotide sequence.

45. A recombinant vector comprising the isolated DNA molecule of claim 21 in a form suitable for transformation of a host cell.

46. An isolated DNA molecule for integrating a polynucleotide sequence including tetracycline-controllable elements (TCE) at a pre-determined location in a target DNA molecule, said isolated DNA molecule comprising the following DNA elements fused in sequence:

a) a first prokaryotic transcription terminator polynucleotide sequence;

b) a second prokaryotic transcription terminator polynucleotide sequence;

c) a polynucleotide sequence encoding a prokaryotic tetracycline resistance protein;

d) a polynucleotide sequence encoding a prokaryotic repressor protein;

e) a first tetracycline-controllable prokaryotic transcription promoter polynucleotide sequence;

f) a second tetracycline-controllable prokaryotic transcription promoter polynucleotide sequence; and

g) a polynucleotide sequence encoding a reporter protein;

said isolated DNA molecule comprising a polynucleotide sequence including the TCE flanked at the end opposite the polynucleotide sequence encoding said reporter protein by additional polynucleotide sequences of sufficient length for homologous

recombination between the isolated DNA molecule and the target DNA molecule at a pre-determined location.

47. A recombinant vector comprising the isolated DNA molecule of claim 46 in a form suitable for transformation of a host cell.

48. A prokaryotic host cell comprising the DNA molecule of claim 46 wherein the DNA molecule is integrated at a pre-determined location in the host cell chromosome.

49. The isolated DNA molecule of claim 46, wherein said recombining elements are comprised of polynucleotides selected from Sequence ID NO: 40, 41, 42 and 43.

50. The isolated DNA molecule of claim 46, wherein said recombining elements are comprised of polynucleotides selected from Sequence ID NO: 44 and 45.

51. The isolated DNA molecule of claim 46, wherein said tetracycline-controllable element is comprised of polynucleotide Sequence ID NO: 37.

52. The isolated DNA molecule of claim 46, further comprising a polynucleotide sequence encoding a reporter gene operably linked to said tetracycline-controllable element.

53. The isolated DNA molecule of claim 46, wherein said tetracycline-controllable element is comprised of polynucleotide Sequence ID NO: 37.

54. The isolated DNA molecule of claim 46, wherein said reporter gene is beta-lactamase.

55. The isolated DNA molecule of claim 46, wherein said reporter gene is beta-lactamase, selected from SEQ ID NO: 38 and 39.

56. The isolated DNA molecule of claim 46, wherein the tetracycline repressor protein is derived from the *Staphylococcus aureus tetM* gene.

57. The isolated DNA molecule of claim 46, wherein the tetracycline repressor protein is derived from the *Staphylococcus aureus tetM* gene selected from SEQ ID NO: 34.

58. The isolated DNA molecule of claim 46, wherein the tetracycline repressor is a *tetR* gene derived from the Tn10 transposon and selected from SEQ ID NO: 35 and 36.

59. An isolated DNA molecule comprising a tetracycline-controllable transcription promoter polynucleotide sequence operably linked to a microbial gene.

60. The isolated DNA molecule of claim 59, wherein said tetracycline-controllable element is comprised of polynucleotide Sequence ID NO: 37.

61. The isolated DNA molecule of claim 60, further comprising a polynucleotide sequence encoding a reporter gene operably linked to said tetracycline-controllable element.

62. The isolated DNA molecule of claim 61, wherein said reporter gene is beta-lactamase.

63. The isolated DNA molecule of claim 62, wherein said reporter gene is beta-lactamase selected from SEQ ID NO: 38 and 39.

64. The isolated DNA molecule of claim 59, further comprising a polynucleotide sequence encoding a a tetracycline resistance (or protection) and repressor DNA cassette (TRRDC) operably linked to a transcription promoter polynucleotide sequence.

65. The isolated DNA molecule of claim 64, further comprising a polynucleotide sequence encoding a prokaryotic a tetracycline resistance (or protection) and repressor DNA cassette (TRRDC), operably linked to a transcription promoter polynucleotide sequence.

66. The isolated DNA molecule of claim 65, wherein said tetracycline resistance (or protection) and repressor DNA cassette (TRRDC) is derived from a *Staphylococcus aureus tetM* gene.

67. The isolated DNA molecule of claim 66, wherein said tetracycline resistance (or protection) and repressor DNA cassette (TRRDC) is derived from the *Staphylococcus aureus tetM* gene comprised of SEQ ID NO: 34.

68. The isolated DNA molecule of claim 67, wherein said tetracycline resistance (or protection) and repressor DNA cassette (TRRDC) is a TN10 derived tetracycline repressor.

69. The isolated DNA molecule of claim 68, wherein the a tetracycline resistance (or protection) and repressor DNA cassette (TRRDC) is a TN10 derived tetracycline repressor selected from the polynucleotides of SEQ ID NO: 35 and 36.

70. A recombinant vector comprising the isolated DNA molecule of claim 21 in a form suitable for transformation of a host cell.

71. A host cell comprising the recombinant vector of claim 70.

72. A recombinant vector comprising the isolated DNA molecule of claim 46 in a form suitable for transformation of a host cell.

73. A host cell comprising the recombinant vector of claim 72

74. A recombinant vector comprising the isolated DNA molecule of claim 59 in a form suitable for transformation of a host cell.

75. A host cell comprising the recombinant vector of claim 74.

76. A process to regulate expression of an endogenous prokaryotic gene comprising the cultivation of the prokaryotic cell in medium with a controlled amount of tetracycline or a tetracycline analog.

77. A process to regulate expression of an endogenous prokaryotic gene comprising the cultivation of the prokaryotic cell in a mammalian host with a controlled amount of tetracycline or a tetracycline analog.

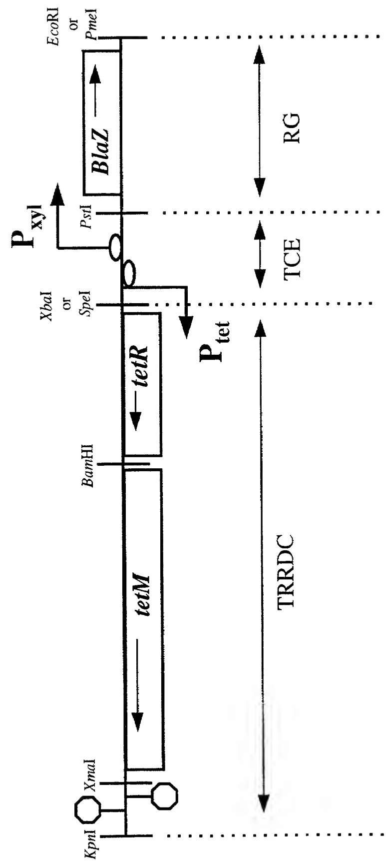
EcoRI
or
PmeI
BlaZ
RG
PstI
P_{xyl}
TCE
XbaI
or
SpeI
P_{tet}
tetR
BamHI
tetM
TRRDC
XmaI
KpnI

Fig. 1

Fig. 2

```
                        sarA terminator
KpnI  ------------>        <------------  terminator
ggtaccgaagtttgatagatgatacattctattaaacttcctttttttat
|---------|---------|---------|---------|--------- 50
ccatggcttcaaactatctactatgtaagataatttgaaggaaaaaaata


gctctgaaaaaacaatgattatctaccttattagtgcagatagataacca
|---------|---------|---------|---------|--------- 100
cgagactttttgttactaataatagatggaataatcacgtctatctattggt
 terminator---------------->        <------------
                         pcrB terminator

         XmaI
ttgtttatcccggg
|---------|--- 114
aacaaatagggccc
------
```

SEQ. I.D. 33

Fig. 3

XmaI
cccgggtaggacacaatatccacttgtagtttataataacgatctcctcc 50
tttccactttaattcaaatctatattaaagaatatttcatcttatttaat 100
aagaaaccatatttatataacaacataaaacgcactaagttattttattg 150
aacatatatcttactttatctatccgactatttagacgacgggtctggca 200
aacaggttcgccagtggtaacctgatatccttttagctctgctaaacaaa 250
cactaagcccatttgtaaaaaaagttaaatcattgcgataatcttgaata 300
catcgagcaggaatttctccaataataatgacctcattatttttcagttg 350
agtatttacgatatttgcacaatatttgggagcatcgttatatgcccgtg 400
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aattctgttccagcttttctaaaggcttgctccagtacaataggagtaag 500
catccgaaaatctgctggagtactaacagggctatagtataaaccgtact 550
taaaacagattttacaatccgtcacattccaaccatataatccttgttcg 600
caaccatagcgtatcccttccataactgcattttgaaatgattgatttaa 650
gtatccaagagaaaccgagctctcatactgcattccacttcccaacggaa 700
gcggtgatacagataaaccaatggaagcccagaaaggatttggcggcact 750
tcgatgtgaatggtatattctgcatttttttaacggtctctccatataaat 800
gactgtaggctctttttagttctatctccacatgatacttttcttgcaaca 850
gtgcactaatcacttccatttgtactttccctaagaaagaaagtataatt 900
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atttgcaaaataacaatttctccagaataagctctatcaatcttacata 1150
attcaccatttattgaagtatacatttctgtaacttttattttttctttt 1200
tctgatactctaaccgaatctcgtaaatgtagtactccactataaaggcg 1250
tatatatgcaagacgttgtctttttttttgtatattcaattttgaaaacat 1300
ttccgcaaagttcagacggacctcgatgtgttgatgaataaaatttatta 1350
gtaataacttctataaggttatcaatccctatattacttttttgcacttcc 1400
atgataaagagggaacagagaacaattctgaaatcttatgctttcctctt 1450
gttcgagttccaatgcttctaatgatttaccggacatatatttctctaaa 1500
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gttcgtcacacacatattaggatacagttctaccttctgtttgattacaa 1600
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tctgacgttctaaaagcgtattatccgtcctcgttgtacctttgtccacg 1950
cttcctaattctgtaatcgctccactgttatataataagctttctgttaa 2000
ggtagtttttcctgcatcaacatgagctaaaactccaatattaataattt 2050
tcatgtgattttcctccatt**ggatcc** 2076
BamHI

SEQ. I.D. 34

Fig. 4A

BamHI

ggatccttaagacccactttcacatttaagttgtttttctaatccgcata 50
tgatcaattcaaggccgaataagaaggctggctctgcaccttggtgatca 100
aataattcgatagcttgtcgtaataatggcggcatactatcagtagtagg 150
tgtttccctttcttctttagcgacttgatgctcttgatcttccaatacgc 200
aacctaaagtaaaatgccccacagcgctgagtgcatataatgcattctct 250
agaaaaaccttgttggcataaaaaggctaattgattttcgagagtttcat 300
actgtttttctgtaggccgtgtacttttgctccatcgcgatgacttagta 350
aagcacatctaaaacttttagcgttattacgtaaaaaatcttgccagctt 400
tccccttctaaagggcaaaagtgagtatggtgcctatctaacatctcaat 450
ggctaaggcgtcgagcaaagcccgcttatttttacatgccaatacaatg 500
taggctgctctacacctagcttctgggcgagtttacgggttgttaaacct 550
tcgattccgacctcattaagcagctctaatgcgctgttaatcactttact 600
tttatctaa**tctaga** 615

XbaI

SEQ. I.D. 35

5/14

Fig. 4B

BamHI

ggatccttaagacccactttcacatttaagttgtttttctaatccgcata 50
tgatcaattcaaggccgaataagaaggctggctctgcaccttggtgatca 100
aataattcgatagcttgtcgtaataatggcggcatactatcagtagtagg 150
tgtttccctttcttctttagcgacttgatgctcttgatcttccaatacgc 200
aacctaaagtaaaatgccccacagcgctgagtgcatataatgcattctct 250
agaaaaaccttgttggcataaaaaggctaattgattttcgagagtttcat 300
actgtttttctgtaggccgtgtacttttgctccatcgcgatgacttagta 350
aagcacatctaaaacttttagcgttattacgtaaaaaatcttgccagctt 400
tccccttctaaagggcaaaagtgagtatggtgcctatctaacatctcaat 450
ggctaaggcgtcgagcaaagcccgcttatttttacatgccaatacaatg 500
taggctgctctacacctagcttctgggcgagtttacgggttgttaaacct 550
tcgattccgacctcattaagcagctctaatgcgctgttaatcactttact 600

XbaI

tttatctaa**tctaga**catcattaattcctaatttttgttgacgacactct 650
atcattgatagagttatttgtcaa**actagt** 680
SpeI

SEQ. I.D. 36

Fig. 5

```
XbaI                                                       tetO
tctagacatcattaattcctcctttttgttgacACTCTATCATTGATAGA
|---------|---------|---------|---------|---------   50
agatctGTAgtaattaaggaggaaaaacaactgTGAGATAGTAACTATCT
    <-Met                                    -10
                                                   Ptet

                SpeI
GTtatttgtcaaactagtttttatttgtcgagttcatgaaaaactaaaa
|---------|---------|---------|---------|---------  100
CAataaacagtttgatcaaaaaataaacagctcaagtactttttgatttt
       -35

                Pxyl
    -35                     -10                 PstI
aaaattgacACTCTATCATTGATAGAGTataattaaaataaaaaagctgc
|---------|---------|---------|---------|---------  150
ttttaactgTGAGATAGTAACTATCTCAtattaattttattttttcgacg
                   tetO

ag
|- 152
tc
```

SEQ. I.D. 39

Fig. 6A

```
PstI
ctgcagcggagggtttattttgaaaaagttaatatttttaattgtaattg   50
ctttagttttaagtgcatgtaattcaaacagttcacatgccaaagagtta  100
aatgatttagaaaaaaatataatgctcatattggtgtttatgctttaga  150
tactaaaagtggtaaggaagtaaaatttaattcagataagagatttgcct  200
atgcttcaacttcaaaagcgataaatagtgctattttgttagaacaagta  250
ccttataataagttaaataaaaaagtacatattaacaaagatgatatagt  300
tgcttattctcctattttagaaaaatatgaggaaaagatatcactttaaa  350
agcacttattgaggcttcaatgacatatagtgataatacagcaaacaata  400
aaattataaaagaaatcggtggaatcaaaaaagttaaacaacgtctaaaa  450
gaactaggagataaagtaacaaatccagttagatatgagatagaattaaa  500
ttactattcaccaaagagcaaaaaagatacttcaacacctgctgctttcg  550
gtaagactttaaataaacttatcgcaaatggaaaattaagcaaagaaaac  600
aaaaaattcttacttgatttaatgttaaataataaaagcggagatacttt  650
aattaaagacggtgttccaaaagactataaggttgctgataaaagtggtc  700
aagcaataacatatgcttctagaaatgatgttgcttttgtttatcctaag  750
ggccaatctgaacctattgttttagtcatttttacgaataaagacaataa  800
aagtgataagccaaatgataagttgataagtgaaaccgccaagagtgtaa  850
tgaaggaattttaagaattcgcatgc                          876
                  EcoRI SphI
```

SEQ. I.D. 38

Fig. 6B

PstI

```
ctgcagcggagggtttattttgaaaaagttaatatttttaattgtaattg   50
ctttagttttaagtgcatgtaattcaaacagttcacatgccaaagagtta  100
aatgatttagaaaaaaatataatgctcatattggtgtttatgctttaga  150
tactaaaagtggtaaggaagtaaaatttaattcagataagagatttgcct  200
atgcttcaacttcaaaagcgataaatagtgctattttgttagaacaagta  250
ccttataataagttaaataaaaaagtacatattaacaaagatgatatagt  300
tgcttattctcctattttagaaaaatatgaggaaaagatatcactttaaa  350
agcacttattgaggcttcaatgacatatagtgataatacagcaaacaata  400
aaattataaaagaaatcggtggaatcaaaaaagttaaacaacgtctaaaa  450
gaactaggagataaagtaacaaatccagttagatatgagatagaattaaa  500
ttactattcaccaaagagcaaaaaagatacttcaacacctgctgctttcg  550
gtaagactttaaataaacttatcgcaaatggaaaattaagcaaagaaaac  600
aaaaaattcttacttgatttaatgttaaataataaaagcggagatacttt  650
aattaaagacggtgttccaaaagactataaggttgctgataaaagtggtc  700
aagcaataacatatgcttctagaaatgatgttgcttttgtttatcctaag  750
ggccaatctgaacctattgttttagtcatttttacgaataaagacaataa  800
aagtgataagccaaatgataagttgataagtgaaaccgccaagagtgtaa  850
tgaaggaattttaagtttaaac                                 872
```

PmeI

SEQ. I.D. 39

Fig. 7A

SacI

cagctctttcagaaatttcggttatgcaacatcattacgttcaaacactc 50
aaggtcgcggtacttacactatgtacttcgatcactatgctgaagttcca 100
aaatcaatcgctgaagatattatcaagaaaaataaaggtgaataatataa 150
cttgttttgactagctagcctaggttaaaatacaaggtgagcttaaatgt 200
aagctatcatctttatagtttgatttttttggggtgaatgcattataaaag 250
aattgtaaaattctttttgcatcgctataaataatttctcatgatggtga 300
gaaactatcatgagagataaattt**ggtacc** 330

KpnI

SEQ. I.D. 40

Fig. 7B

PmeI
gtttaaacgaataggagagattttataatggcaaaagaaaaattcgatcg 50
ttctaaagaacatgccaattcggtacttcggtcacgttgaccatggtaaa 100
acaacattaacagcaatcgctactgtattagcaaaaaatggtgactcagt 150
tgcacaatcatatgacatgattgacaacgctccagaagaaaaagaacgtg 200
gtatcacaatcaatacttctcacattgagtaccaaactgacaaacgtcac 250
tacgctcacgttgactgcccaggacacgctgactacgttaaaaacatgat 300
cactggtgctgctcaaatggacggcggtatcttagtagtatctgctgctg 350
acggtccaatgccacaaactcgt**gaattcgcatgc** 385
EcoRI SphI

SEQ. I.D. 41

Fig. 8A

SacI

gagctcggttgcagatggcattgtcattggtagcgaaatcgttaagcgat 50
ttaaatctaacacgcgtgaggaaatcattaaatatttacaatctatccaa 100
caaacattgaataattaagtttacttgatttaaaaaaattaggcgaatac 150
tgtttgaaaaagtgaaaaacggtgaattataaaattgaatacaatttcaa 200
aaaaagtaatatgagcaaacccaaacgttcatattactttttttgaaatt 250
gtattcaaaaatctaaatattactataaaagtatacgcaattaaagcgtt 300
tatgttttagttttaacattaactattgtatacttatttagattagattt 350
attattttgacatttgcagagg**ggtacc** 379

KpnI

SEQ. I.D. 42

Fig. 8B

```
  PmeI
gtttaaactgcaaatacggaaatgaaattaattaacgagagacaaatagg   50
agtaatgataatgaagtttacaaatttaacagctaaagagtttggtgcct  100
ttacagatagcatgccatacagtcatttcacgcaaactgttggccactat  150
gagttaaagcttgctgaaggttatgaaacacatttagtgggaataaaaaa  200
caataataacgaggtcattgcagcttgcttacttactgctgtacctgtta  250
tgaaagtgttcaagtatttttattcaaatcgcggtccagtgattgattat  300
gaaaatcaagaactcgtacactttttctttaatgaattatcaaaatatgt  350
taaaaaacatcgttgtctatacctacatatcgatccatatttaccatatc  400
aatacttgaattcgcatgcg                                420
       EcoRI  SphI
```

SEQ. I.D. 43

Fig. 9A

SacI

gagctcgggttcaatattaactgaaaaagaattagattaaatattaattt 50
ggaaaactggaacaaccaaaaagttatatgaccgcgtaggtcttaatgaa 100
gagacgctaagtattttagatactgaaatcactaaaaaaacaatacctgt 150
aagacctggtagaaatgttgcggtaattattgaggtcgctgcaatgaact 200
atcgattaaatatcatgggcattaacactgccgaagaatttagtgaaaga 250
ttaaatgaagaaattatcaagaacagtcataaga**ggtacc** 290

KpnI

SEQ. I.D. 44

Fig. 9B

PmeI

```
gtttaaacggaggagtaggttgaatgggtattgtatttaactatatagat   50
cctgtggcatttaacttaggaccactgagtgtacgatggtatggaattat  100
cattgctgtcggaatattacttggttactttgttgcacaacgtgcactag  150
ttaaagcaggattacataaagatactttagtagatattatttttttatagt  200
gcactatttggatttatcgcggcacgaatctattttgtgattttccaatg  250
gccatattacgcggaaaatccaagtgaaattattaaaatatggcatggtg  300
gaatagcaatacatggtggtttaataggtggctttattgctggtgttatt  350
gtatgtaaaggaaaaatttaaacccatttcaaattggtgatatcgttgcg  400
ccaagtataattttagcgcaaggaattcgcatgc                  434
```

EcoRI SphI

SEQ. I.D. 45

DECLARATION (37 CFR §1.63) AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, and

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **AN AUTOREGULATORY SYSTEM FOR VALIDATING MICROBIAL GENES AS POSSIBLE ANTIMICROBIAL TARGETS USING A TETRACYCLINE-CONTROLLABLE ELEMENT**, Docket No. **6137.P US**, the specification of which

[] is attached hereto.

[] was filed on as Application Serial No. and was amended on .

[X] was filed on 12 January 1999 as PCT International Application No. PCT/US99/00371 and was amended under PCT Article 19 on , if applicable.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with 37 CFR §1.56(a).

I hereby claim the benefit under 35 USC §119(e) of any United States provisional application(s) listed below:

Application Serial No.	Filing Date (Day/Month/Year)
60/071,640	16 January 1998

Information to be completed if claiming priority of PROVISIONAL APPLICATION(s)

I hereby claim foreign priority benefits under 35 USC §119(a)-(d), or §365(b), of any foreign application(s) for patent or inventor's certificate or §365(a) of any PCT International Application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT International Application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application on which priority is claimed:

Application Serial No.	Country	Filing Date (Day/Month/Year)	Priority Claimed (Yes/No)

Information to be completed if claiming priority of PROVISIONAL APPLICATION(s)

I hereby claim the benefit under 35 USC §120, of any United States application(s) or PCT International Application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application(s) in the manner provided by the first paragraph of 35 USC §112, I acknowledge the duty to disclose material information as defined in 37 CFR §1.56(a), which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (Patented, Pending, Abandoned)

Information to be completed if claiming priority of PROVISIONAL APPLICATION(s)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint Carl W. Battle (Registration No. 30,731); James D. Darnley, Jr. (Registration No. 33,673); Ellen K. Park (Registration No. 34,055); Bruce A. Pokras (Registration No. 32,748); Edward F. Rehberg (Registration No. 34,703); Andrew M. Solomon (Registration No. 32,175); Bruce Stein (Registration No. 27,231); Thomas A. Wootton (Registration No. 35,004); and Lucy X. Yang (Registration No. 40,259); Ann M. Mueting (Reg. No. 33,977), Kevin W. Raasch (Reg. No. 35,651), Mark J. Gebhardt (Reg. No. 35,518), Victoria A. Sandberg (Reg. No. 41,287), David L. Provence (Reg. No. 43,022), Matthew W. Adams (Reg. No. 43,459), and Loren D. Albin (Reg. No. 37,763); all registered to practice before the Patent and Trademark Office as my attorneys or agents with full power of substitution and revocation to prosecute this application and all divisions and continuations thereof and to transact all business in the Patent and Trademark Office connected therewith and request that all correspondence and telephone communications be directed to the following person at the mailing address and telephone number hereafter given:

Name	: Ann M. Mueting, Attorney
Registration No.	: 33,977
Address	: Mueting, Raasch & Gebhardt, P.A. P.O. Box 581415 Minneapolis, Minnesota 55458-1415 United States of America
Telephone No.	: (612) 305-1217
Telefax No.	: (612) 305-1228

1-00

Inventor's Signature Charles W. Ford Date 7/11/2000

Full name of sole or first inventor: Charles W. Ford
Residence: Portage, MI 49024 MI Citizenship: USA
Post Office Address: 1306 Carriage, Portage, MI 49024

2-00

Inventor's Signature Cheryl L. Quinn Date 7-12-2000

Full name of second joint inventor: Cheryl L. Quinn
Residence: Kalamazoo, MI 49008 MI Citizenship: USA
Post Office Address: 3010 Ridgeview Circle, Kalamazoo, MI 49008

SEQUENCE LISTING

<110> Quinn, Cheryl L.
Ford, Charles W.
Pharmacia & Upjohn Company

<120> An Autoregulatory Tetracycline-Regulated System for
Validating Bacterial Genes as Antibacterial Targets

<130> 6137

<140> 6137
<141> 1998-01-16

<160> 45

<170> PatentIn Ver. 2.0

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<210> 3

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<223> Description of Artificial Sequence: Synthetic
      Oligonucleotides

<400> 3

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<210> 4

<211> 28

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<213> Artificial Sequence

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      Oligonucleotides

<400> 4

ggggtacccg ctgaagagat agcgattg 28

<210> 5

<211> 33

<212> DNA

<213> Artificial Sequence

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Oligonucleotides

<400> 5

acgcacgagc tctttcagaa atgttcggtt atg 33

<210> 6

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

<400> 6

ggggtaccaa atttatctct catgatag 28

<210> 7

<211> 21

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Oligonucleotides

<400> 7

caggtacagc agtaagtaag c 21

<210> 8

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic Oligonucleotides

<400> 8

gtcaacgtga gcgtagtgac g 21

<210> 9

<211> 54

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Synthetic Oligonucleotides

<400> 9

cgaagtttga tagatgatac attctattaa acttcctttt tttatgctct gaaa 54

<210> 10

<211> 50

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<213> Artificial Sequence

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aaacaatgat tatctacctt attagtgcag atagataacc attgtttatc 50

<210> 11

<211> 52

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Oligonucleotides

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<211> 60

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Oligonucleotides

<400> 12

ccgggataaa caatggttat ctatctgcac taataaggta gataatcatt gttttttcag 60

<210> 13

<211> 27

<212> DNA

<213> Artificial Sequence

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<400> 13

cgggatccaa tggaggaaaa tcacatg 27

<210> 14

<211> 33

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Synthetic Oligonucleotides

<400> 14

tccccccggg taggacacaa tatccacttg tag 33

<210> 15

<211> 39

<212> DNA

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<400> 15

gactagtttg acaaataact ctatcaatga tagagtgtc 39

<210> 16

<211> 26

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<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

<400> 16
taatgatgtc tagattagat aaaagt 26

<210> 17
<211> 29
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<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

<400> 17
cgggatcctt aagacccact ttcacattt 29

<210> 18
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Oligonucleotides

<400> 18
ctagacatca ttaattcctc ctttttgttg acactctatc attgatagag ttatttgtca 60
aa 62

<210> 19
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<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

<400> 19

ctagtttgac aaataactct atcaatgata gtgtcaacaa aaaggaggaa ttaatgatgt 60

<210> 20

<211> 46

<212> DNA

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<223> Description of Artificial Sequence: Synthetic
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ctagtttttt atttgtcgag ttcatgaaaa actaaaaaaa attgac 46

<210> 21

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<400> 21

tttttttag tttttcatga actcgacaaa taaaaaa 37

<210> 22

<211> 42

<212> DNA

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Oligonucleotides

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actctatcat tgatagagta taattaaaat aaaaaagctg ca 42

<210> 23

<211> 40

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Oligonucleotides

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acatacgcat gcgaattctt aaaattcctt cattacactc 40

<210> 24

<211> 43

<212> DNA

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<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

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gctttttat tttaattata ctctatcaat gatagagtgt caa 43

<210> 25

<211> 30

<212> DNA

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<223> Description of Artificial Sequence: Synthetic Oligonucleotides

<400> 25

aactgcagta atatcggagg gtttattttg 30

<210> 26

<211> 29

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<223> Description of Artificial Sequence: Synthetic Oligonucleotides

<400> 26

gtttaaactt aaaattcttc attacactc 29

<210> 27

<211> 42

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Synthetic Oligonucleotides

<400> 27

ggaattttaa gtttaaactg caaatacgga aatgaaatta at 42

<210> 28

<211> 41

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<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

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acatacgcat gcgaattcaa gtattgatat ggtaaatatg g 41

<210> 29

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

<400> 29

ggaattttaa gtttaaacga ggagtaggtt gaatgggta 39

<210> 30

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Snythetic

Oligonucleotides

<400> 30

acatacgcat gcgaattcct tgcgctaaaa ttatac 36

<210> 31

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Snythetic

Oligonucleotides

<400> 31

ggaattttaa gtttaaacga ataggagaga ttttataatg gc 42

<210> 32

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Snythetic

Oligonucleotides

<400> 32

acatacgcat gcgaattcac gagtttgtgg cattggacc 39

<210> 33

<211> 114

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA
fragment

<400> 33

ggtaccgaag tttgatagat gatacattct attaaacttc cttttttat gctctgaaaa 60

aacaatgatt atctacctta ttagtgcaga tagataacca ttgtttatcc cggg 114

<210> 34

<211> 2076

<212> DNA

<213> Escherichia coli

<400> 34

cccgggtagg acacaatatc cacttgtagt ttataataac gatctcctcc tttccacttt 60

aattcaaatc tatattaaag aatatttcat cttatttaat aagaaaccat atttatataa 120

caacataaaa cgcactaagt tattttattg aacatatatc ttactttatc tatccgacta 180

tttagacgac gggtctggca aacaggttcg ccagtggtaa cctgatatcc ttttagctct 240

gctaaacaaa cactaagccc atttgtaaaa aaagttaaat cattgcgata atcttgaata 300

catcgagcag gaatttctcc aataataatg acctcattat ttttcagttg agtatttacg 360

atatttgcac aatatttggg agcatcgtta tatgcccgtg aaagatattc ctgtggtgca 420

taaactttaa aactaagata tggctctaac aattctgttc cagcttttct aaaggcttgc 480

tccagtacaa taggagtaag catccgaaaa tctgctggag tactaacagg gctatagtat 540

aaaccgtact taaaacagat tttacaatcc gtcacattcc aaccatataa tccttgttcg 600

caaccatagc gtatcccttc cataactgca ttttgaaatg attgatttaa gtatccaaga 660

gaaaccgagc tctcatactg cattccactt cccaacggaa gcggtgatac agataaacca 720

atggaagccc agaaaggatt tggcggcact tcgatgtgaa tggtatattc tgcatttttt 780

aacggtctct ccatataaat gactgtaggc tcttttagtt ctatctccac atgatacttt 840

tcttgcaaca gtgcactaat cacttccatt tgtactttcc ctaagaaaga aagtataatt 900

tcatgtgtcg tagaatccac gtaatatcgt agaagcggat cactatctga gatttccaaa 960

agggcatcaa gcaacatttc tctctgttca ggtttactcg gttcaacagt tgtttgtagt 1020

agagggtgcg gattttcaat ctttttttctc tgtggcaata gttttgtatc tccaagaaca 1080

ctatttaact tcaaaaactc attttgcaaa ataacaattt ctccagaata agctctatca 1140

atcttacata attcaccatt tattgaagta tacatttctg taacttttat tttttctttt 1200

tctgatactc taaccgaatc tcgtaaatgt agtactccac tataaaggcg tatatatgca 1260
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ctttcctctt gttcgagttc caatgcttct aatgatttac cggacatata tttctctaaa 1500
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cacatattag gatacagttc taccttctgt ttgattacaa tttcggcaga aagtttctct 1620
ttaatatcct gataaaccgt tgataaatca attccatttt ggtcaatctt attgataaaa 1680
aagattgtgg gaatccccat tttcctaagt gcatgaaata atatacgagt ttgtgcttgt 1740
acgaaatctt ttgcagaaat cagtagaatt gccccatcta aaactgataa tgaacgatat 1800
acttctgcta agaaatccat atgtcctggc gtgtctatga tgttcacctt cgtattttcc 1860
cactgaaaag aggttattcc tgtctgaatt gtaattcctc tctgacgttc taaaagcgta 1920
ttatccgtcc tcgttgtacc tttgtccacg cttcctaatt ctgtaatcgc tccactgtta 1980
tataataagc tttctgttaa ggtagttttt cctgcatcaa catgagctaa aactccaata 2040
ttaataattt tcatgtgatt ttcctccatt ggatcc 2076

<210> 35

<211> 615

<212> DNA

<213> Escherichia coli

<220>

<223> Description of Combined DNA/RNA Molecule: Nucleic acid

<400> 35

ggatccttaa gacccacttt cacatttaag ttgtttttct aatccgcata tgatcaattc 60
aaggccgaat aagaaggctg gctctgcacc ttggtgatca aataattcga tagcttgtcg 120
taataatggc ggcatactat cagtagtagg tgtttccctt tcttctttag cgacttgatg 180
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aaaactttta gcgttattac gtaaaaaatc ttgccagctt tccccttcta aagggcaaaa 420
gtgagtatgg tgcctatcta acatctcaat ggctaaggcg tcgagcaaag cccgcttatt 480

ttttacatgc caatacaatg taggctgctc tacacctagc ttctgggcga gtttacgggt 540

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tttatctaat ctaga 615

<210> 36

<211> 680

<212> DNA

<213> Escherichia coli

<400> 36

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taataatggc ggcatactat cagtagtagg tgtttccctt tcttctttag cgacttgatg 180

ctcttgatct tccaatacgc aacctaaagt aaaatgcccc acagcgctga gtgcatataa 240

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actgtttttc tgtaggccgt gtacttttgc tccatcgcga tgacttagta aagcacatct 360

aaaactttta gcgttattac gtaaaaaatc ttgccagctt tccccttcta aagggcaaaa 420

gtgagtatgg tgcctatcta acatctcaat ggctaaggcg tcgagcaaag cccgcttatt 480

ttttacatgc caatacaatg taggctgctc tacacctagc ttctgggcga gtttacgggt 540

tgttaaacct tcgattccga cctcattaag cagctctaat gcgctgttaa tcactttact 600

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gagttatttg tcaaactagt 680

<210> 37

<211> 152

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
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gatagagtat aattaaaata aaaaagctgc ag 152

<210> 38

<211> 876

<212> DNA

<213> Staphylococcus aureus

<400> 38

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taatgctcat attggtgttt atgctttaga tactaaaagt ggtaaggaag taaaatttaa 180

ttcagataag agatttgcct atgcttcaac ttcaaaagcg ataaatagtg ctattttgtt 240

agaacaagta ccttataata agttaaataa aaaagtacat attaacaaag atgatatagt 300

tgcttattct cctattttag aaaaatatga ggaaaagata tcactttaaa agcacttatt 360

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aagagtgtaa tgaaggaatt ttaagaattc gcatgc 876

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<211> 872

<212> DNA

<213> Staphylococcus aureus

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taatgctcat attggtgttt atgctttaga tactaaaagt ggtaaggaag taaaatttaa 180

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<213> Staphylococcus aureus

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tacttacact atgtacttcg atcactatgc tgaagttcca aaatcaatcg ctgaagatat 120
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gaaactatca tgagagataa atttggtacc 330

<210> 41
<211> 385
<212> DNA
<213> Staphylococcus aureus

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tactgtatta gcaaaaaatg gtgactcagt tgcacaatca tatgacatga ttgacaacgc 180

tccagaagaa aaagaacgtg gtatcacaat caatacttct cacattgagt accaaactga 240

caaacgtcac tacgctcacg ttgactgccc aggacacgct gactacgtta aaaacatgat 300

cactggtgct gctcaaatgg acggcggtat cttagtagta tctgctgctg acggtccaat 360

gccacaaact cgtgaattcg catgc 385

<210> 42

<211> 379

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<213> Staphylococcus aureus

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ttacttgatt taaaaaaatt aggcgaatac tgtttgaaaa agtgaaaaac ggtgaattat 180

aaaattgaat acaatttcaa aaaaagtaat atgagcaaac ccaaacgttc atattacttt 240

ttttgaaatt gtattcaaaa atctaaatat tactataaaa gtatacgcaa ttaaagcgtt 300

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acatttgcag aggggtacc 379

<210> 43

<211> 420

<212> DNA

<213> Staphylococcus aureus

<400> 43

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agtcatttca cgcaaactgt tggccactat gagttaaagc ttgctgaagg ttatgaaaca 180

catttagtgg gaataaaaaa caataataac gaggtcattg cagcttgctt acttactgct 240

gtacctgtta tgaaagtgtt caagtatttt tattcaaatc gcggtccagt gattgattat 300

gaaaatcaag aactcgtaca ctttttcttt aatgaattat caaaatatgt taaaaaacat 360

cgttgtctat acctacatat cgatccatat ttaccatatc aatacttgaa ttcgcatgcg 420

<210> 44

<211> 290

<212> DNA

<213> Staphylococcus aureus

<400> 44

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aacaaccaaa aagttatatg accgcgtagg tcttaatgaa gagacgctaa gtattttaga 120

tactgaaatc actaaaaaaa caatacctgt aagacctggt agaaatgttg cggtaattat 180

tgaggtcgct gcaatgaact atcgattaaa tatcatgggc attaacactg ccgaagaatt 240

tagtgaaaga ttaaatgaag aaattatcaa gaacagtcat aagaggtacc 290

<210> 45

<211> 434

<212> DNA

<213> Staphylococcus aureus

<400> 45

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ttaacttagg accactgagt gtacgatggt atggaattat cattgctgtc ggaatattac 120

ttggttactt tgttgcacaa cgtgcactag ttaaagcagg attacataaa gatactttag 180

tagatattat tttttatagt gcactatttg gatttatcgc ggcacgaatc tattttgtga 240

ttttccaatg gccatattac gcggaaaatc caagtgaaat tattaaaata tggcatggtg 300

gaatagcaat acatggtggt ttaataggtg gctttattgc tggtgttatt gtatgtaaag 360

gaaaaattta aacccatttc aaattggtga tatcgttgcg ccaagtataa ttttagcgca 420

aggaattcgc atgc 434

SEQUENCE LISTING

<110> PHARMACIA & UPJOHN COMPANY
Quinn, Cheryl L.
Ford, Charles W.

<120> AN AUTOREGULATORY SYSTEM FOR VALIDATING MICROBIAL GENES AS POSSIBLE ANTIMICROBIAL TARGETS USING A TETRACYCLINE-CONTROLLABLE ELEMENT

<130> 6137.P US

<140> Unassigned
<141> 2000-07-13

<150> 60/071,640
<151> 1998-01-16

<160> 45

<170> PatentIn Ver. 2.1

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<220>
<223> Description of Artificial Sequence: Synthetic Oligonucleotides

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acgcacgagc tcggttgcag atggcattgt c 31

<210> 2
<211> 26
<212> DNA
<213> Artificial Sequence

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<400> 2
ggggtacccc ctctgcaaat gtcaaa 26

<210> 3
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

<400> 3
acgcacgagc tcagatcttc gcttgtgcgg 30

<210> 4
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

<400> 4
ggggtacccg ctgaagagat agcgattg 28

<210> 5
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

<400> 5
acgcacgagc tctttcagaa atgttcggtt atg 33

<210> 6
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

<400> 6
ggggtaccaa atttatctct catgatag 28

<210> 7
<211> 21
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Oligonucleotides

<400> 7
caggtacagc agtaagtaag c 21

<210> 8
<211> 21
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<213> Artificial Sequence

<220>
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Oligonucleotides

<400> 8
gtcaacgtga gcgtagtgac g 21

<210> 9
<211> 54
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

<400> 9
cgaagtttga tagatgatac attctattaa acttcctttt tttatgctct gaaa 54

<210> 10
<211> 50
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotides

<400> 10
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<210> 11
<211> 52
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotides

<400> 11
agcataaaaa aaggaagttt aatagaatgt atcatctatc aaacttcggt ac   52

<210> 12
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotides

<400> 12
ccgggataaa caatggttat ctatctgcac taataaggta gataatcatt gtttttcag 60

<210> 13
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotides

<400> 13
cgggatccaa tggaggaaaa tcacatg   27

<210> 14

<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotides

<400> 14
tccccccggg taggacacaa tatccacttg tag 33

<210> 15
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotides

<400> 15
gactagtttg acaaataact ctatcaatga tagagtgtc 39

<210> 16
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotides

<400> 16
taatgatgtc tagattagat aaaagt 26

<210> 17
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotides

<400> 17

cgggatcctt aagacccact ttcacattt 29

<210> 18
<211> 62
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

<400> 18
ctagacatca ttaattcctc ctttttgttg acactctatc attgatagag ttatttgtca 60
aa 62

<210> 19
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

<400> 19
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<210> 20
<211> 46
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

<400> 20
ctagtttttt atttgtcgag ttcatgaaaa actaaaaaaa attgac 46

<210> 21
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotides

<400> 21
tttttttag tttttcatga actcgacaaa taaaaaa 37

<210> 22
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotides

<400> 22
actctatcat tgatagagta taattaaaat aaaaaagctg ca 42

<210> 23
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotides

<400> 23
acatacgcat gcgaattctt aaaattcctt cattacactc 40

<210> 24
<211> 43
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotides

<400> 24
gctttttat tttaattata ctctatcaat gatagagtgt caa 43

<210> 25

<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotides

<400> 25
aactgcagta atatcggagg gtttattttg   30

<210> 26
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotides

<400> 26
gtttaaactt aaaattcttc attacactc   29

<210> 27
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotides

<400> 27
ggaattttaa gtttaaactg caaatacgga aatgaaatta at   42

<210> 28
<211> 41
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotides

<400> 28

acatacgcat gcgaattcaa gtattgatat ggtaaatatg g 41

<210> 29
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

<400> 29
ggaattttaa gtttaaacga ggagtaggtt gaatgggta 39

<210> 30
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

<400> 30
acatacgcat gcgaattcct tgcgctaaaa ttatac 36

<210> 31
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

<400> 31
ggaattttaa gtttaaacga ataggagaga ttttataatg gc 42

<210> 32
<211> 39
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
      Oligonucleotides

<400> 32
acatacgcat gcgaattcac gagtttgtgg cattggacc 39

<210> 33
<211> 114
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA
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<400> 33
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aacaatgatt atctacctta ttagtgcaga tagataacca ttgtttatcc cggg 114

<210> 34
<211> 2076
<212> DNA
<213> Escherichia coli

<400> 34
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caacataaaa cgcactaagt tattttattg aacatatatc ttactttatc tatccgacta 180
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<210> 35
<211> 615
<212> DNA
<213> Escherichia coli

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taataatggc ggcatactat cagtagtagg tgtttccctt tcttctttag cgacttgatg 180
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actgtttttc tgtaggccgt gtacttttgc tccatcgcga tgacttagta aagcacatct 360
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<210> 36
<211> 680
<212> DNA
<213> Escherichia coli

<400> 36
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taataatggc ggcatactat cagtagtagg tgtttccctt tcttctttag cgacttgatg 180
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aaaactttta gcgttattac gtaaaaaatc ttgccagctt tccccttcta aagggcaaaa 420
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gtgagtatgg tgcctatcta acatctcaat ggctaaggcg tcgagcaaag cccgcttatt 480
ttttacatgc caatacaatg taggctgctc tacacctagc ttctgggcga gtttacgggt 540
tgttaaacct tcgattccga cctcattaag cagctctaat gcgctgttaa tcactttact 600
tttatctaat ctagacatca ttaattccta atttttgttg acgacactct atcattgata 660
gagttatttg tcaaactagt 680

<210> 37
<211> 152
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Oligonucleotides

<400> 37
tctagacatc attaattcct ccttttgtt gacactctat cattgataga gttatttgtc 60
aaactagttt tttatttgtc gagttcatga aaaactaaaa aaaattgaca ctctatcatt 120
gatagagtat aattaaaata aaaaagctgc ag 152

<210> 38
<211> 876
<212> DNA
<213> Staphylococcus aureus

<400> 38
ctgcagcgga gggtttattt tgaaaaagtt aatattttta attgtaattg ctttagtttt 60
aagtgcatgt aattcaaaca gttcacatgc caaagagtta aatgatttag aaaaaaaata 120
taatgctcat attggtgttt atgctttaga tactaaaagt ggtaaggaag taaaatttaa 180
ttcagataag agatttgcct atgcttcaac ttcaaaagcg ataaatagtg ctattttgtt 240
agaacaagta ccttataata agttaaataa aaaagtacat attaacaaag atgatatagt 300
tgcttattct cctattttag aaaaatatga ggaaaagata tcactttaaa agcacttatt 360
gaggcttcaa tgacatatag tgataataca gcaaacaata aaattataaa agaaatcggt 420
ggaatcaaaa aagttaaaca acgtctaaaa gaactaggag ataaagtaac aaatccagtt 480
agatatgaga tagaattaaa ttactattca ccaaagagca aaaaagatac ttcaacacct 540
gctgctttcg gtaagacttt aaataaactt atcgcaaatg gaaaattaag caaagaaaac 600
aaaaaattct tacttgattt aatgttaaat aataaaagcg gagatacttt aattaaagac 660
ggtgttccaa aagactataa ggttgctgat aaaagtggtc aagcaataac atatgcttct 720
agaaatgatg ttgcttttgt ttatcctaag ggccaatctg aacctattgt tttagtcatt 780
tttacgaata aagacaataa aagtgataag ccaaatgata agttgataag tgaaaccgcc 840
aagagtgtaa tgaaggaatt ttaagaattc gcatgc 876

<210> 39
<211> 872
<212> DNA

<213> Staphylococcus aureus

<400> 39
ctgcagcgga gggtttattt tgaaaaagtt aatattttta attgtaattg ctttagtttt 60
aagtgcatgt aattcaaaca gttcacatgc caaagagtta aatgatttag aaaaaaaata 120
taatgctcat attggtgttt atgctttaga tactaaaagt ggtaaggaag taaaatttaa 180
ttcagataag agatttgcct atgcttcaac ttcaaaagcg ataaatagtg ctattttgtt 240
agaacaagta ccttataata agttaaataa aaaagtacat attaacaaag atgatatagt 300
tgcttattct cctattttag aaaaatatga ggaaaagata tcactttaaa agcacttatt 360
gaggcttcaa tgacatatag tgataataca gcaaacaata aaattataaa agaaatcggt 420
ggaatcaaaa aagttaaaca acgtctaaaa gaactaggag ataaagtaac aaatccagtt 480
agatatgaga tagaattaaa ttactattca ccaaagagca aaaaagatac ttcaacacct 540
gctgctttcg gtaagacttt aaataaactt atcgcaaatg gaaaattaag caaagaaaac 600
aaaaaattct tacttgattt aatgttaaat aataaaagcg gagatacttt aattaaagac 660
ggtgttccaa aagactataa ggttgctgat aaaagtggtc aagcaataac atatgcttct 720
agaaatgatg ttgcttttgt ttatcctaag ggccaatctg aacctattgt tttagtcatt 780
tttacgaata aagacaataa aagtgataag ccaaatgata agttgataag tgaaaccgcc 840
aagagtgtaa tgaaggaatt ttaagtttaa ac 872

<210> 40
<211> 330
<212> DNA
<213> Staphylococcus aureus

<400> 40
cagctctttc agaaatttcg gttatgcaac atcattacgt tcaaacactc aaggtcgcgg 60
tacttacact atgtacttcg atcactatgc tgaagttcca aaatcaatcg ctgaagatat 120
tatcaagaaa aataaaggtg aataatataa cttgttttga ctagctagcc taggttaaaa 180
tacaaggtga gcttaaatgt aagctatcat ctttatagtt tgatttttttg gggtgaatgc 240
attataaaag aattgtaaaa ttctttttgc atcgctataa ataatttctc atgatggtga 300
gaaactatca tgagagataa atttggtacc 330

<210> 41
<211> 385
<212> DNA
<213> Staphylococcus aureus

<400> 41
gtttaaacga ataggagaga ttttataatg gcaaaagaaa aattcgatcg ttctaaagaa 60
catgccaatt cggtacttcg gtcacgttga ccatggtaaa acaacattaa cagcaatcgc 120
tactgtatta gcaaaaaatg gtgactcagt tgcacaatca tatgacatga ttgacaacgc 180
tccagaagaa aaagaacgtg gtatcacaat caatacttct cacattgagt accaaactga 240
caaacgtcac tacgctcacg ttgactgccc aggacacgct gactacgtta aaaacatgat 300
cactggtgct gctcaaatgg acggcggtat cttagtagta tctgctgctg acggtccaat 360
gccacaaact cgtgaattcg catgc 385

<210> 42
<211> 379
<212> DNA
<213> Staphylococcus aureus

<400> 42
gagctcggtt gcagatggca ttgtcattgg tagcgaaatc gttaagcgat ttaaatctaa 60
cacgcgtgag gaaatcatta aatatttaca atctatccaa caaacattga ataattaagt 120
ttacttgatt taaaaaaatt aggcgaatac tgtttgaaaa agtgaaaaac ggtgaattat 180
aaaattgaat acaatttcaa aaaaagtaat atgagcaaac ccaaacgttc atattacttt 240
ttttgaaatt gtattcaaaa atctaaatat tactataaaa gtatacgcaa ttaaagcgtt 300
tatgttttag ttttaacatt aactattgta tacttattta gattagattt attatttttg 360
acatttgcag aggggtacc 379

<210> 43
<211> 420
<212> DNA
<213> Staphylococcus aureus

<400> 43
gtttaaactg caaatacgga aatgaaatta attaacgaga gacaaatagg agtaatgata 60
atgaagttta caaatttaac agctaaagag tttggtgcct ttacagatag catgccatac 120
agtcatttca cgcaaactgt tggccactat gagttaaagc ttgctgaagg ttatgaaaca 180
catttagtgg gaataaaaaa caataataac gaggtcattg cagcttgctt acttactgct 240
gtacctgtta tgaaagtgtt caagtatttt tattcaaatc gcggtccagt gattgattat 300
gaaaatcaag aactcgtaca ctttttcttt aatgaattat caaaatatgt taaaaaacat 360
cgttgtctat acctacatat cgatccatat ttaccatatc aatacttgaa ttcgcatgcg 420

<210> 44
<211> 290
<212> DNA
<213> Staphylococcus aureus

<400> 44
gagctcgggt tcaatattaa ctgaaaaaga attagattaa atattaattt ggaaaactgg 60
aacaaccaaa aagttatatg accgcgtagg tcttaatgaa gagacgctaa gtattttaga 120
tactgaaatc actaaaaaaa caatacctgt aagacctggt agaaatgttg cggtaattat 180
tgaggtcgct gcaatgaact atcgattaaa tatcatgggc attaacactg ccgaagaatt 240
tagtgaaaga ttaaatgaag aaattatcaa gaacagtcat aagaggtacc 290

<210> 45
<211> 434
<212> DNA
<213> Staphylococcus aureus

<400> 45
gtttaaacgg aggagtaggt tgaatgggta ttgtatttaa ctatatagat cctgtggcat 60
ttaacttagg accactgagt gtacgatggt atggaattat cattgctgtc ggaatattac 120
ttggttactt tgttgcacaa cgtgcactag ttaaagcagg attacataaa gatactttag 180
tagatattat tttttatagt gcactatttg gatttatcgc ggcacgaatc tattttgtga 240
ttttccaatg gccatattac gcggaaaatc caagtgaaat tattaaaata tggcatggtg 300
gaatagcaat acatggtggt ttaataggtg gctttattgc tggtgttatt gtatgtaaag 360
gaaaaattta aacccatttc aaattggtga tatcgttgcg ccaagtataa ttttagcgca 420
aggaattcgc atgc 434